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Nutritional programming of gilthead sea bream for better utilisation of low fish meal and fish oil diets

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Chapter 1

GENERAL INTRODUCTION

1.1 Importance of n-3 LC-PUFA in human nutrition

Research on the importance of n-3 long-chain polyunsaturated fatty acids with 20 or more carbon atoms (LC-PUFA) in human nutrition has a long history. The effects of dietary n-3 LC-PUFA have been extensively studied in humans. Even though there are contradictory opinions, yearly over 1000 publications evidence the importance of n-3 LC-PUFA, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), for human health. n-3 LC-PUFA have been found to play an important role in prevention of cardiovascular diseases (CVD), hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders, and even some types of cancers (Simopoulos, 2000; Calder, 2018). According to the World Health Organisation, CVD such as, ischemic heart disease and stroke are among the world's largest causes of death, accounting for a combined 15.2 million deaths (approximately 28% of all deaths) in 2016. (World Health Organisation, 2018). There is an abundance of literature showing the beneficial effects of LC-PUFA consumption for prevention of CVD, despite some studies have not been able to establish a clear relation between n-3 LC-PUFA treatment and CVD mortalities (Abdelhamid *et al.*, 2018). Some terrestrial foods such as walnuts, flaxseed oil, soybean, or rapeseed oil are rich in 18C fatty acids, precursors of n-3 LC-PUFA, such as α-linolenic acid (ALA, 18:3n-3). However, none of them have considerable amounts of n-3 LC-PUFA (Whelan and Rust, 2006). Human beings have a certain capacity to synthesize the n-3 LC-PUFA from their 18C precursor ALA (Domenichiello *et al.*, 2015). However, this capacity is restricted. For instance, the conversation ratio of ALA to EPA is calculated to be 0.2% for EPA and even lower than 0.1% for DHA (22:6n-3). However, this ratio could be as high as 6% in EPA in pregnant females which believed to meet pregnancy demands (Burdge, 2006). As well as gender, this conversion ratios

also can be influenced by variety of other factors such as age, hormones and genetic background (Bolton-Smith *et al.*, 1997; Vessby *et al.*, 2002; Koletzko *et al.*, 2010).

In most geographical regions, marine derived products, particularly oily-fish, are the only source of LC-PUFA and, hence, fish consumption is being endorsed by the different health authorities (SACN, 2004; Health and Services, 2015). For instance, UK health authorities recommends a daily consumption of 450 mg n-3 LC-PUFA. While a portion of 100 g chicken supplies only 0.06 g EPA + DPA + DHA, the same quantity of salmon supplies 2.2 grams of these fatty acids (Calder, 2018). Since at present one of every two aquatic products consumed worldwide are coming from aquaculture, this food production sector is carrying a main responsibility in supplying n-3 LC-PUFA for the consumers demand. Despite aquaculture produced fish has until now a larger content on n-3 LC-PUFA than wild fish, since fillet fatty acid profiles are dependent on the fish diet, it is very important to supply these diets with these valuable fatty acids. However, fish oil (FO) is the main, but non-sustainable, source of n-3 LC-PUFA in aquafeeds and its replacement by vegetable oils (VO) may negatively affect the nutritional value of fish filet for human consumption (Izquierdo *et al.*, 2003, Castro *et al.*, 2015; Izquierdo *et al.*, 2005; Torstensen *et al.*, 2010).

1.2 Importance of n-3 LC-PUFA in fishes

1.2.1 Functions

Essential fatty acids (EFA) in fish nutrition play important roles in metabolic processes along the life-cycle of the animal. For instance, fatty acids are sources of energy for the metabolic progresses and are a preferred source of energy for growth, reproduction and swimming (Tocher, 2003). They are major sources of energy for fish from very early stages of development to adult fish (Tocher *et al.*, 1985; Tocher *et al.*, 1985). Their roles in fish reproduction success is well-established in many fish species, including gilthead sea bream (*Sparus aurata*) (Izquierdo *et al.*, 2001). Lipids are not only important for reproduction, but also for providing energy for the offspring, through the yolk sac for the development of the embryo. Deficiency of n-3 LC-PUFA levels may lead lower fertilization rates in broodstock (Izquierdo *et al.*, 2001), partly in relation to reduced

sperm mobility (Vassallo-Agius *et al.*, 2001). Yolk sac composition also determines the embryonic and early larval stages of gilthead sea bream until mount opening, since individuals solely relies on the nutrients supplied by the yolk sac. In gilthead sea bream, free amino acids are responsible for 60-70% of the energy supply during embryogenesis, while fatty acids are the most important (80-90%) source of energy after hatching (Rønnestad *et al.*, 1994). It is important to note that, in particular, neutral lipids are a preferred energy source for the larvae after the hatching.

Fatty acids are structural components of phospholipids, a major component of all cell membranes (Izquierdo, 1996). Moreover, in specific tissues, such as neural ones, especially DHA (22:6n-3) is important for the proper development and functioning of brain and eye (the retina and nerves related to vision) (Benitez-Santana *et al.*, 2007; Turkmen *et al.*, 2017) during the early life stages. For instance, in gilthead sea bream, during 16 to 26 days after hatch (dah) in late larval developmental stage, survival and growth is decreased if larvae are fed with 16 mg/g n-3 LC-PUFA dietary fatty acid levels in comparison to 73.5 mg/g fed individuals (Turkmen *et al.*, 2017). Besides, the retinal ganglion cells counting showed that increasing dietary DHA (22:6n-3) levels significantly increases the amount of bromodeoxyuridine positive cells in gilthead sea bream. (Turkmen *et al.*, 2017). In general, for broodstock and larval nutrition the most important fatty acids seems to be EPA (20:5n-3), DHA (22:6n-3) and in lesser extend arachidonic acid (ARA, 20:4n-6) (Izquierdo *et al.*, 2001). However, this should not mean these fatty acids should be provided to broodstock and larvae in excess since, for instance (Koven *et al.*, 2001) low ARA (20:4n-6) intake can also improve the stress resistance of gilthead sea bream. On the other hand, the EFA requirements for gilthead sea bream is much higher in during the spawning period. Indeed, in this species, n-3 LC-PUFA requirement changes between 1.5-2.5% of the diets, which is up to 2% higher than juveniles (Izquierdo, 1996).

Some fatty acids are also precursors of bioactive molecules (Ganga *et al.*, 2005). For this reason, dietary fats quantity and quality have a profound effect on immune system functioning (Calder, 1996; Calder, 2002; Yaqoob, 2004; Calder, 2006; Yaqoob

and Calder, 2007). Thus, dietary n-6 fatty acids, high in terrestrial animals and many VO, stimulate the production of pro-inflammatory eicosanoids and cytokines in mammals (Calder, 2006) and fish (Secombes *et al.*, 1996; Montero and Izquierdo, 2010), whereas dietary DHA (22:6n-3) and EPA (20:5n-3) suppress the production of those pro-inflammatory substances (Calder, 2005). Among other physiological mechanisms, inflammation processes are mediated by the activation of phospholipases A2 (PLA2), which release LC-PUFA from membrane phospholipids, and cyclooxygenases (COX), which produce eicosanoids from those fatty acids (Izquierdo and Koven, 2011). Although COX have a great affinity for ARA (20:4n-6) yielding prostaglandins (PG) from 2-series, EPA (20:5n-3) is also good substrate for COX producing 3-series PG and other eicosanoids that are less potent than ARA (22:4n-6) derivatives and play an important anti-inflammatory role, through mediators termed E-series resolvins (Serhan, 2006). DHA-derived d-resolvins also have anti-inflammatory properties (Marcheselli *et al.*, 2003). Thus, ARA (22:4n-6) derived eicosanoids enhance the production of inflammatory cytokines such as tumor necrosis factor alpha (TNF-alfa) and interleukin-1 (IL-1) (Calder, 2006; Montero *et al.*, 2015) that are the first cytokines up-regulated after injuries or infection, whereas EPA (20:5n-3) and DHA (22:6n-3) reduce chronic inflammation by indirectly down-regulating both cytokines (Farooqui *et al.*, 2007). Effects of the LC-PUFA on the recognition of specific antigens or pathogens have been less studied. For instance, the cells surface proteins, major histocompatibility complex I (MHCI) and II (MHCII) are up-regulated by ARA-derived prostaglandins in mammals and fish (Hwang *et al.*, 2000; Torrecillas *et al.*, 2017).

Dietary LC-PUFA markedly improve stress resistance in fish (Montero and Izquierdo, 2010). Thus, in fish larvae, increase in DHA (22:6n-3) and EPA (20:5n-3) improves resistance to handling or thermal acute stress (Liu *et al.*, 2002) and, in juveniles, prevents elevation of cortisol levels during chronic stress (Montero *et al.*, 1998). Thus, cortisol release by head kidney cells after stimulation with adrenal corticotrophin hormone (ACTH) is modulated by PUFA through lysyl oxidase (LOX) products (Ganga *et al.*, 2006). Moreover, these lipids may bind to glucocorticoid receptors (GR) and its gene expression (Montero *et al.*, 2015), modulating the action of cortisol. Besides, LC-PUFA

also regulate the expression of mitochondrial and cytoplasmic chaperons such as 70 kilodalton heat shock proteins (HSP70) and 90 kilodalton heat shock proteins (HSP90), which are necessary to assembly and maintenance of GR, increasing the binding capacity of this steroid receptor (Benedito-Palos *et al.*, 2016).

1.2.2 Metabolism of lipids and n-3 LC-PUFA biosynthesis

Generally speaking, marine fish seem to have a lower ability to utilize terrestrial oils due their high n-3 LC-PUFA requirements and the insufficient activity of enzymes synthesizing n-3 LC-PUFA from the fatty acid precursors found in VO. Thus, substitution of FO with VO seems more challenging in marine fish than in other species that are better equipped to utilize dietary lipids more efficiently. The higher LC-PUFA biosynthesis capacity in freshwater fish in comparison to marine fish could be related to differences in the feeding habits and nutrients intake, with marine fish having a continuous access to LC-PUFA rich sources throughout their lives (Sargent *et al.*, 1995; Tocher, 2003). Besides, these differences among fish species have been related to the diverse evolution of certain genes involved in lipid biosynthesis (Castro *et al.*, 2016). Linoleic acid (LA, 18:2n-6) and ALA (18:3n-3) are the precursors of the n-3 LC-PUFA biosynthesis and can be converted to longer carbon chain fatty acids such as EPA (20:5n-3) and DHA (22:6n-3) after several elongation and desaturation steps (Figure 1-1). The first step of n-3 LC-PUFA synthesis in vertebrates is achieved by fatty acyl delta-6-desaturase enzyme (delta6), encoded by fatty acid desaturase 2 gene (*fads2*), by introducing a double bond in a specific position of long-chain fatty acids. In the first step of this pathway, LA (18:2n-6) and ALA (18:3n-3) can be converted to 18:3n-6 and 18:4n-3, respectively. After an elongation step, these fatty acids can be converted to 20:3n-6 and 20:4n-3. From these precursors, there is another desaturation step catalysed by a fatty acyl delta-5-desaturase enzyme (delta5) involved in the conversion to 20:5n-3 (EPA) and 20:4n-6 (ARA) (Figure 1-1). Up to date, there is only one desaturase gene (*fads2*) isolated from gilthead sea bream and very low delta-5 activity has been found during *in vitro* studies (Tocher and Ghioni, 1999). Fish desaturases have differently evolved in different fish species and show a variety of desaturases (Castro *et al.*, 2016). For instance, in zebrafish

(*Danio rerio*) *fads2* gene gives rise to both delta5 and delta6 activities (Hastings *et al.*, 2001). Under this perspective, some metabolic pathways and the n-3 LC-PUFA synthesis capacity in gilthead sea bream remains unclear. However, with the increasing knowledge and availability of new genomic tools, including the recent publication of the whole gilthead sea bream genome sequence (Pauletto *et al.*, 2018), new opportunities to better understand the n-3 LC-PUFA synthesis mechanisms and their regulation are being opened to answer the metabolic constraints of n-3 LC-PUFA metabolism in gilthead sea bream.

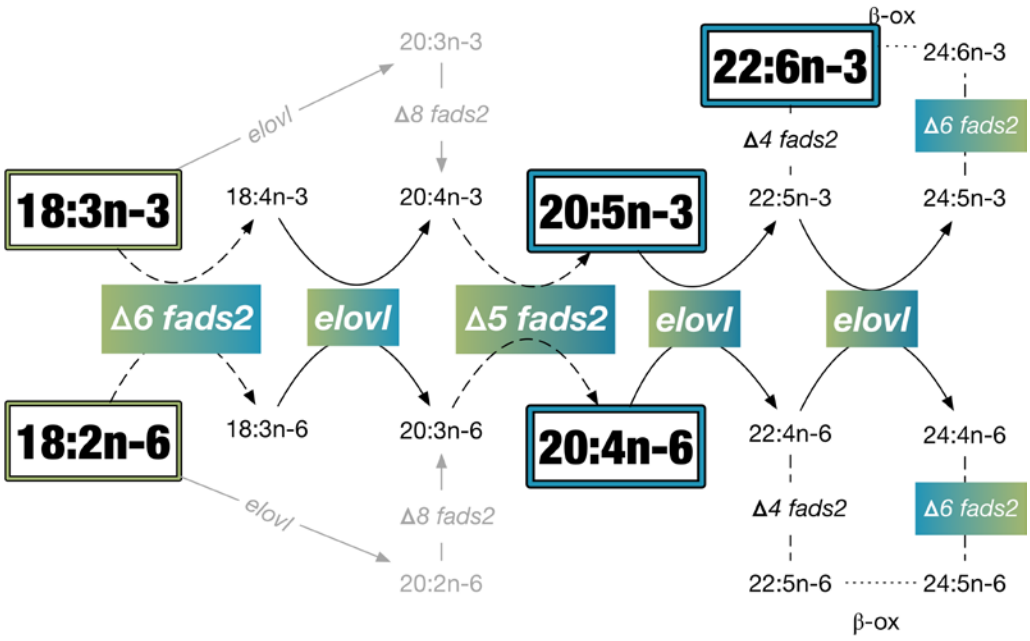

Figure 1-1 | LC-PUFA biosynthesis pathway and the enzymes involved

1.2.3 LC-PUFA metabolism related genes studied in the present thesis

As explained before delta6 activity, a rate-limiting enzyme of the LC-PUFA biosynthesis, is encoded by *fads2* and for this reason this gene is widely studied in variety organisms and fish species. The crucial role of *fads2* is clear in n-3 LC-PUFA biosynthesis however, there are several other enzymes involved in this biosynthesis pathway. For instance, LC-PUFA synthesis also requires chain elongation catalysed by elongases (*elovl*) with different substrate preferences (Monroig *et al.*, 2011). Among them, elongation of very long chain fatty acids protein 6 (*elovl6*) is a key lipogenic enzyme

which elongates long-chain saturated and monounsaturated fatty acids of 12, 14 and 16 carbon atoms, which has received much attention due its importance in metabolic disorders in mammals (Matsuzaka and Shimano, 2009). Besides, LC-PUFA may have a direct effect on the expression of other genes related to lipid or carbohydrate metabolism (Clarke, 2001). Lipoprotein lipase (*lpl*) facilitates the tissue up-take of circulating fatty acids (Bell and Koppe, 2010) from lipoproteins and its expression in the liver can be regulated by n-3 LC-PUFA (Raclot *et al.*, 1997). The provision of energy is accomplished by β-oxidation of free fatty acids transported into the mitochondria in the form of fatty acyl-carnitine esters by carnitine acyltransferases, such as carnitine palmitoyltransferases (Sargent *et al.*, 2002). Replacement of FO with VO changes the fatty acid composition of liver and muscle, affecting the β-oxidation capacity and regulating the expression of carnitine palmitoyltransferase I (*cpti*) and carnitine palmitoyltransferase II (*cptII*) genes (Kjaer *et al.*, 2008; Leaver *et al.*, 2008; Vestergren *et al.*, 2012; Xue *et al.*, 2015). β-oxidation also takes place in the peroxisome and is modulated by peroxisome proliferator activator receptors (PPARs). Three different PPAR isoforms (α, β, γ) have been characterised in gilthead sea bream, peroxisome proliferator-activated receptor alpha (*ppara*) being the major form expressed in the liver (Leaver *et al.*, 2008). PPARs are nuclear receptors that regulate differentiation, growth, and metabolism and in mammals, epigenetic mechanisms have been described to regulate these processes involving all the PPARs isoforms (Corbin, 2011). For instance, feeding pregnant rats a protein-restricted diet reduces methylation of the ppara promoter in the offspring and the hypomethylation persists into adulthood (Lillycrop *et al.*, 2008). Finally, another gene potentially regulated by LC-PUFA is cyclooxygenase-2 (*cox2*), a key enzyme in prostanoids biosynthesis (Ishikawa and Herschman, 2007). The genes used in the present thesis were selected to cover a variety of lipid metabolism related genes (Figure 1-2).

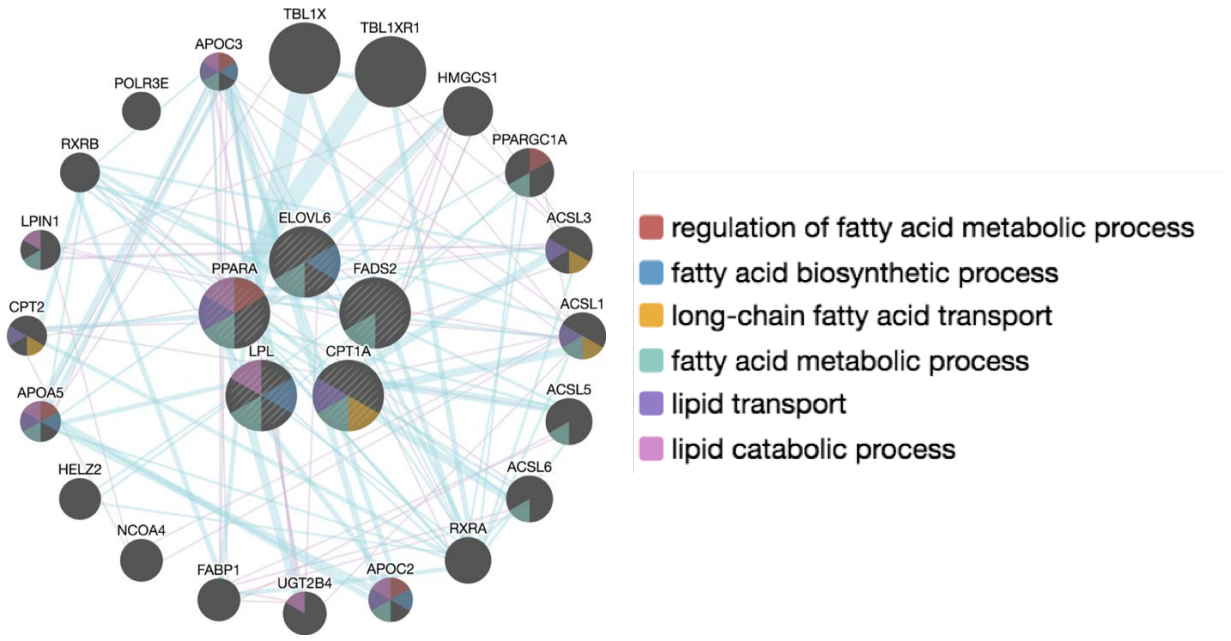

Figure 1-2 | Lipid metabolism related genes and their interaction between different metabolic pathways. Created using genemania.org

1.3 Problems about the fish meal and oil replacement with vegetable meals and oils in aquafeeds

FM and FO production mainly depends on wild fisheries of small oily pelagic fish such as Peruvian anchoveta (*Engraulis ringens*)(Cashion *et al.*, 2017). However, their availability is limited by the natural production of specific fisheries and may vary from year to year, due to overfishing, climate conditions and, even, natural disasters. For instance, El Niño climate process, markedly reduced fishmeal (FM) and FO production, that of 1998 being one of the most remarkable in history. Production of FM and FO (live weight) reached 19.4 million tonnes by 2011, whereas it was reduced to 16.3 million tonnes by 2012 (FAO, 2016). Since the demand of these products is steadily increasing, not only to maintain a higher aquaculture production, but also for many other human activities, the prices are increasing, particularly during years of shortage as those of El Niño. The annual changes in FM, FO and aquaculture production are presented in Figure 1-3. Moreover, there is an increasing demand for FO for human consumption (FAO, 2016). While FO production is steady, increasing demand causes rise on the prices and also affects quality. World Bank statistics show that FO prices have increased from

446.22 Euros/ton in January 2000, to 1165.26 Euros/ton in January 2014, raising almost three times in these 15 years (FAO, 2016). The use of fish by-products to produce FM is increasing trying to fulfil the demand for these ingredients, but its proven to be insufficient to cover aquafeeds requirements. Therefore, decrease in availability and rise of FM and FO prices, has forced the industry to search for more sustainable and inexpensive candidates for both economic and environmental point of view.

Aquaculture is the fastest growing food production sector accounting at present more than 52% of the world consumed fish (FAO, 2018), but a major issue concerning such development is the over dependence of fish feeds on capture fishery-derived raw materials such as FM and FO (Kaushik and Troell, 2010). The established beneficial roles of FO on human health (Wang *et al.*, 2006) and the use of FO, albeit in small proportions, in other animal production systems, have led to an increase in demand for this raw material, consequently raising the prices. Recent research shows that 75% of the FO produced was used by aquaculture, 22% for human consumption and 3% for other usages (Tocher, 2015).

Despite the great achievements to reduce FM in diets for marine fish species (Robaina *et al.*, 1995; Fernández-Palacios *et al.*, 1997; Robaina *et al.*, 1998; Kaushik *et al.*, 2004; Le Boucher *et al.*, 2011; Le Boucher *et al.*, 2013; Shepherd and Bachis, 2014,García-Romero et al., 2014; Torrecillas *et al.*, 2017; Torrecillas *et al.*, 2017), complete replacement of FO remains still a major challenge. Moreover, complete substitution of FO negatively affects immune system and stress and disease resistance (Montero and Izquierdo, 2010) and reduces the fillet content in n-3 LC-PUFA, such as EPA (20:5n-3) and DHA (22:6n-3), negatively affecting the nutritional value of fish flesh for humans (Izquierdo *et al.*, 2005; Rosenlund *et al.*, 2010).

In the recent years, some authors have predicted that the further development of aquaculture will be limited by the FO production (Naylor *et al.*, 2009). Nevertheless, during the last decades, there has been an increasing research activity on substitution of FO by terrestrial sources (Gatlin *et al.*, 2007; Glencross, 2009; Turchini *et al.*, 2009; Eryalçın *et al.*, 2013; Betancor *et al.*, 2016).

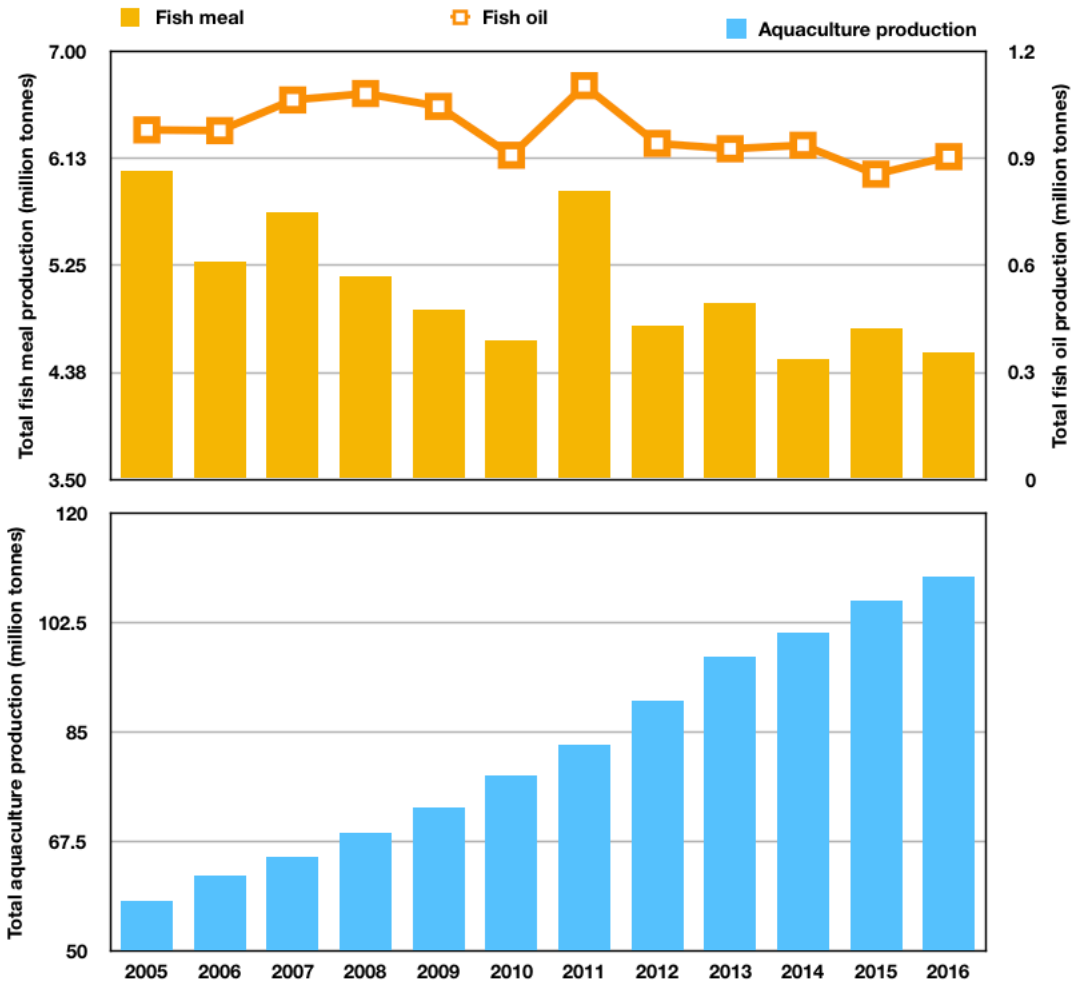

Figure 1-3 | Total production of fish oil, fishmeal and aquaculture production (Statistics obtained from IFFO for FO and FM, FAO 2018 for the total aquaculture production

However, tolerance to VO greatly varies among fish species, and freshwater/cold water ones tend to better utilize terrestrial plant oils than marine fish. For instance, growth rates of rainbow trout (*Oncorhynchus mykiss*) were not affected by total replacement of cod liver oil by corn oil (Arzel *et al.*, 1994) or up to 80% substitution of FO by a mixture of VO (soybean, rapeseed, olive and palm oil) (Caballero *et al.*, 2002). In brown trout (*Salmo trutta*), growth was similar when fish were fed either vegetable oils or animal fats (Turchini *et al.*, 2003). No differences were either found in growth of Atlantic salmon (*Salmo salar*) fed by 100% FO or 100% VO (mixture of rapeseed oil, palm oil, and LO) through the whole production cycle (24 months) (Torstensen *et al.*, 2005). On the contrary, despite growth of European sea bass (*Dicentrachus labrax*) is not affected

by a 60% replacement of FO by soybean oil, LO, rapeseed oil or a mixture of these oils if the minimum EFA requirements are covered (Izquierdo *et al.*, 2003), higher FO substitution levels tend to reduce fish growth (Montero *et al.*, 2005) and negatively affect fish health (Montero and Izquierdo, 2010). Many other studies demonstrate that complete FO replacement affects growth in many carnivorous fish such as red sea bream (*Pagrus auratus*) (Glencross *et al.*, 2003; Glencross *et al.*, 2003), sharpsnout sea bream (*Diplodus puntazzo*) (Almaida-Pagan *et al.*, 2007; Piedecausa *et al.*, 2007), Senegalese sole (*Solea senegalensis*) (Benitez-Dorta *et al.*, 2013) or orange-spotted grouper (*Epinephelus coioides*) (Lin *et al.*, 2007).

Similarly, gilthead sea bream growth is not affected by 60% FO replacement with soybean oil, linseed oil (LO), rapeseed oil or mixture of these oils, if the minimum content of EFA are provided to fulfil the requirements (i.e. including small quantities of FM) (Izquierdo *et al.*, 2003). However further substitution by 80% soybean or LO resulted on a reduced growth (Izquierdo, 2005). Other studies corroborate this findings and complete FO replacement by VO mixtures (17:25:58 % of rapeseed: palm: linseed oils) in diets with low FM content markedly reduced fish growth (Benedito-Palos *et al.*, 2007). Complete FO replacement with 100% LO, 100% soybean oil or a 50/50 mixture of these VO resulted in decreased growth in gilthead seabream juveniles (Montero *et al.*, 2008).

1.4 Nutritional programming concept in vertebrates and mammals

Nutritional programming is described as the “events during critical or sensitive periods of development that may “program” long-term or life-time structure or function of the organism” (Lucas, 1998). The “nutritional programming” concept presumes that alterations of the environment during the early life stages of an organism affect its phenotype at later-life stages. Environmental changes during critical early periods, such as embryogenesis or early development (referred as developmental plasticity periods), provide a tool to the organism to forecast potential environmental challenges in later life and give the opportunity to adjust its metabolism for a better adaptation increasing its chances to survive and reproduce. In a way, these critical periods of developmental plasticity allow the organism to prepare itself for its future environment. Such long-term

outcomes may be addressed in three different ways: direct damage; induction, deletion or impaired development of a somatic structure resulting from a stimulus or insult during a critical period; physiological "setting'' by an early stimulus or insult at a critical period, with long-term consequences for function. For instance, the offspring that faced calorie restrictions during the fetal growth may adopt a phenotype described as "thrifty phenotype" later in life, such as permanent changes in glucose-insulin metabolism (Hales and Barker, 2001).

There are several evidences that show how the organism prepares itself if it confronts energy restrictions during fetal development. One of the first evidences of the linkage between the early nutrition and its long-term effects in human is the well-known effects of the Dutch Famine of World War II. During this food blockage in the Netherlands, calorie intake of the individuals dropped from 1800 kcal/day in December 1943, below to 1000 kcal in late November and to only 400 to 800 kcal/day by the April 1945. Thus, during his period, together with the rest of the population, pregnant women faced a severe famine that imposed mandatory calorie restrictions to their embryos during their early stages of life. Well-kept records during World War II allowed researchers to investigate the effects of this famine and the relation between poor maternal nutrition and offspring health. In addition, researchers today were able to study different parameters in their offspring. These available data provide an almost perfectly designed human experiment in the effects of maternal nutrition restriction and the later outcomes in the progeny. This study was one of the first examples for the growing field of the developmental origins of health and disease in humans, which show that inadequate nutrition during the developmental processes may have different metabolic consequences in the offspring via epigenetic mechanisms (Barella *et al.*, 2017)(Figure 1-4).

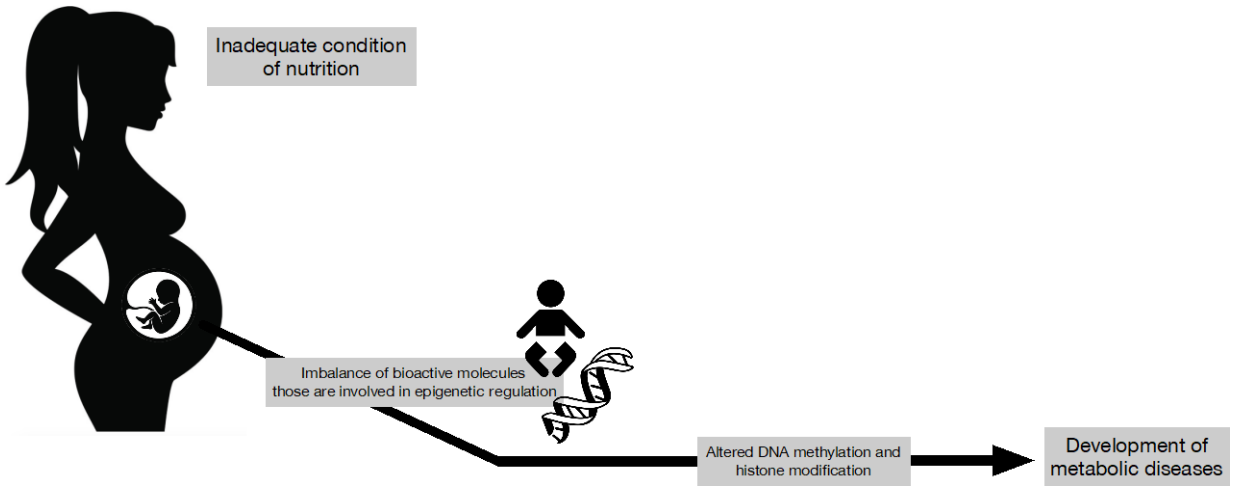

Figure 1-4 I Inadequate nutrition during the developmental processes may develop metabolic consequences in the offspring via epigenetic mechanisms in humans, re-drawn from (Barella *et al.*, 2017)

Another pragmatic example of how parental nutrition affects progenies performance is the agouti mouse. In this mouse model, if the maternal mouse feeding with diets rich in methyl donors such as choline, betaine, folic acid, vitamin B_{12}, L-methionine or zinc before breeding and through pregnancy triggers a specific phenotype and metabolic changes in the offspring. This is a popular example in the field of epigenetic research, since not only has health and metabolism consequences, but also resulted in a distinctive coat colour (Wolff *et al.*, 1998). This coat colour is depending on the modulation of the so-called agouti gene, which encodes a peptide responsible for the production of a yellow pigment in the coat. If this gene is expressed the mice develop a yellow coat, whereas silenced agouti gene expression results in a brown coat colour. Those yellow-coloured individuals were obese, prone to diabetes, more susceptible develop tumours, and lived shorter in comparison to their genetically identical brown-coloured siblings (Wolff *et al.*, 1998). This study was the basis in agouti mouse for identifying the underlying epigenetic mechanisms, such as deoxyribonucleic acid (DNA) methylation in developing embryos. Thus, changes in metabolism and coat colour of agouti mice are mediated by changes in the DNA methylation pattern of the agouti gene (Waterland and Jirtle, 2003).

Another of the best-known examples of nutritional programming occurs in western honeybees (*Apis melifera*), where genetically identical females when fed with

royal jelly turn into queen bees with different morphology and reproductive properties (Colhoun and Smith, 1960). This type of nutrition during the larval stages change the phenotype of the animal for the rest of its life. Again, DNA methylation during very early larval development is the underlying mechanism for this differentiation as it has been more recently demonstrated (Kucharski *et al.*, 2008).

Apart from these examples, an increasing number of studies show that environmental factors experienced by the parents can have long-lasting effects in the offspring or in the later generations (Burdge *et al.*, 2007; Burton and Metcalfe, 2014), due to their importance in prevention of metabolic disorders in humans (Langley-Evans, 2015). Particularly, during reproduction environmental signs can markedly modulate metabolic routes and offspring phenotype (Gluckman and Hanson, 2004; Burdge *et al.*, 2007; Curley *et al.*, 2011). The potential mechanisms for storing the nutritional programming event until adulthood include adaptive changes in gene expression (epigenetic effect: DNA methylation, histone modification, micro ribonucleic acid (miRNA)), preferential clonal selection of adapted cells in programmed tissues and programmed differential proliferation of tissue cell types (Waterland and Jirtle, 2003; Symonds and Budge, 2009) (Figure 1-5).

These adaptational changes can be used in favour to obtain individuals better adapted to the feeds used in animal production systems. In production systems, diets are formulated to supply the necessary nutritional components, selecting appropriate ingredients based on their nutritional characteristics, cost and availability. Thus, restrictions in the availability of a high-quality feedstuff lead to the search for alternative ingredients.

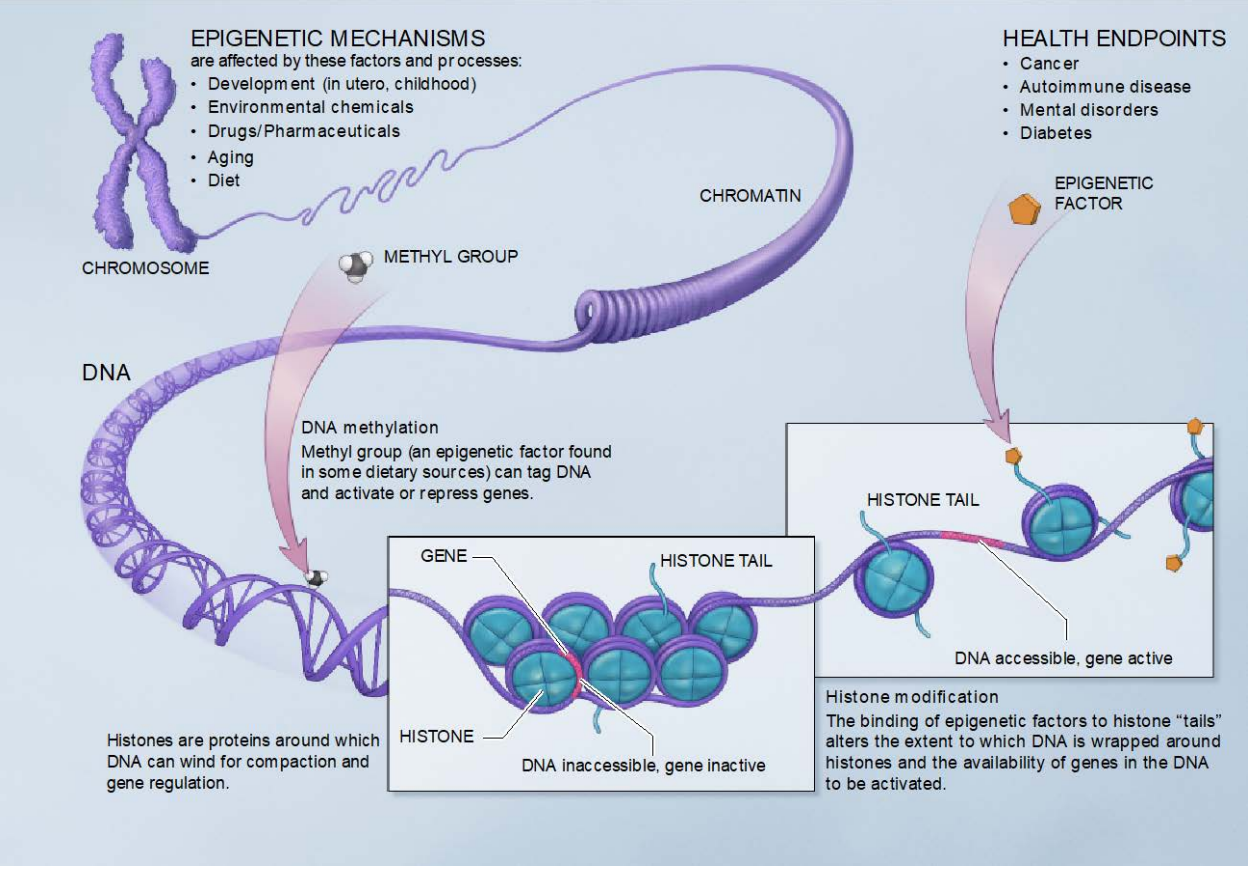

Figure 1-5 I Epigenetic mechanisms involved in regulation of gene expression – adapted from National Institutes of Health http://commonfund.nih.gov/epigenomics/figure

For instance, fish have evolved along evolution to prey in certain types of organisms, adapting their metabolism to the use of the nutrients these preys' content. Based on this premises, diets in aquaculture were formulated as close as possible to those preys to supply the necessary nutrients to achieve optimal growth and health-status. Therefore, traditionally, FM and FO obtained from capture-fisheries have been used in diets to respectively fulfil protein and lipid requirements of marine fish. Unfortunately, capture-derived sources cannot supply the demand of the aquaculture sector to sustain its rapid development (detailed in section 1.3). However, replacement of FM and FO by terrestrial sources is restricted by different types of problems, including the low levels or even absolutely lack of certain nutrients such as essential fatty acids.

Consequently, there has been an increasing interest in aquaculture to find out different ways to maximize the capacity of fish to use alternative sources in a species

dependent manner. For instance, in rainbow trout, one of the major species in European freshwater aquaculture with 814 thousand tonnes (2% of word total aquaculture production (FAO, 2018), there has been an intensive effort to increase its capacity to utilize terrestrial feed ingredients (Geurden *et al.*, 2007; Geurden *et al.*, 2013; Fontagné-Dicharry *et al.*, 2017; Mellery *et al.*, 2017; Michl, 2017; Panserat *et al.*, 2019). Carbohydrates are in general an economic source of energy in formulated diets for animal species. Being rainbow trout a glucose-intolerant species at the highest trophic level of the food chain, it has a very low capacity to utilize carbohydrates (Skiba-Cassy *et al.*, 2013). Since at first feeding expression of genes involved in glucose metabolism may be modulated (Marandel *et al.*, 2016), a short-time stimulus (3 days) during this period with a hyperglucidic diet influences carbohydrate digestion later in life (10 g) when juveniles are challenged for 1 week with a high carbohydrate diet (Geurden *et al.*, 2007). This short-time stimuli at first feeding causes the up-regulation of α-amylase, maltase gastrointestinal or hepatic gene expression in 1.5-1.7 folds than the control group fed with commercial diets low in carbohydrates through the first feeding period) (Geurden *et al.*, 2007). Moreover, if the swim-up fry fish is fed with a FM/FO devoted diet for 3 weeks are switch back to the commercial diet, later in life at the juvenile stages (7-months-age) they showed a better acceptance of plant-based diets (30%) than juveniles without a nutritional programming history (Geurden *et al.*, 2013). In addition to the better acceptance of the diets, juveniles showed 42% higher growth and 18% improved feed utilization in comparison to those that did not received a nutritional intervention with the FM/FO devoted diet (Geurden *et al.*, 2013). In another example, zebra fish (*Danio rerio*), a model species for research, supplementation of egg yolk with glucose showed little regulatory effects on the carbohydrate metabolism in later stages (Rocha *et al.*, 2014).

In marine fish, complete replacement of FO is still a main challenge in aquaculture diets, due to their low ability to synthesize n-3 LC-PUFA and the lack of these fatty acids in vegetable oils. For this reason, studies on nutritional programming have also aimed to increase marine fish capacity to utilize low n-3 LC-PUFA diets. In European sea bass, early studies established that nutritional programming through larval feeding with a diet deficient in n-3 long-chain polyunsaturated fatty acids with 18 or more carbon atoms

persistently affects the expression of the gene codifying for a LC-PUFA biosynthesis rate-limiting enzyme, the fatty acid desaturase 2 gene (*fads2*), when juveniles were later challenged with a low n-3 LC-PUFA diet (Vagner *et al.*, 2009). In these studies, European sea bass larvae were fed either a high or low LC-PUFA diet (0.8% eicosapentaenoic acid (EPA, 20:5n-3) plus docosahexaenoic acid (DHA, 22:6n-3) or 2.2% EPA+DHA) from 6–45 dah (Vagner *et al.*, 2007). Afterwards, all fish groups were fed same commercial diet until 151 dah. Then, all juveniles were nutritionally challenged with a LC-PUFA deprived diet for 2 months, the growth of those fed a low LC-PUFA diet at larval stages growing faster than those fed with high LC-PUFA diet at larval stages (Vagner *et al.*, 2007). In addition, *fads2* gene expression in liver was up-regulated in juveniles that were fed a low LC-PUFA diet during larval stages (Vagner *et al.*, 2007). Moreover, feeding European sea bass larvae with four different levels of n-3 LC-PUFA (extra-high, high, low and extra-low n-3 LC-PUFA), *fads2* gene expression in juveniles was negatively correlated to the n-3 LC-PUFA contents in their livers (Vagner *et al.*, 2007). Besides, there were no differences in growth or total fatty EPA (20:5n-3) and DHA (22:6n-3) content of the whole body of the juveniles after the challenge test (Vagner *et al.*, 2007). However, the mechanisms behind the modulation of *fads2* in juveniles by larval feeding with different dietary lipids were not yet clarified.

Nevertheless, similar studies conducted with gilthead sea bream showed adverse nutritional programming effects in juveniles. For instance, nutritional interventions during the early developmental period (3-16 dah) through live prey enrichment has negative consequences in larval growth and survival rates and negative programming effect on the fish in juvenile stages when challenged with vegetable meal (VM) / vegetable oil (VO) based diets (Izquierdo *et al.*, in prep). Moreover, nutritional stimuli during the late larval developmental stage (16-30 dah) feeding larvae with VO source diet also caused very high mortalities that did not allow to produce sufficient progeny to challenge fish at juvenile stage with low FM/FO diets (Turkmen *et al.*, 2017).

1.5 Dietary lipids in early nutritional programming through parental nutrition in mammals and fish

Lipids in early nutrition of mammals, especially through parental nutrition, have received considerable scholarly attention in recent years, partly due to the changes in consumption of lipid types and amounts observed in western populations (Foundations, 1994). These changes imply an increase in lipid consumption, particularly saturated fatty acids, that have been linked to a higher occurrence of CVD and the metabolic syndrome (Reddy, 2002). Increasing number of evidences found between the early exposures to dietary interventions and the modulation of the metabolism in the offspring channelled some research effort on the programming effects of lipids in humans. Indeed, extensive research, frequently using rodents as models (Vandamme, 2014) have shown the linkage between early-environmental clues and the future risk of disease and metabolic changes (Gluckman *et al.*, 2008), For instance, fat levels and sources in pregnant and lactating rats induce different metabolic effects in the offspring (Kelsall *et al.*, 2012). To investigate these effects, maternal rats were fed diets with either 7 or 21% lipids high in either linoleic acid (LA, 18:2n-6, from safflower oil), trans-fatty acids (from hydrogenated soybean oil), saturated fatty acids (from butter) or n-3 LC-PUFA (from FO), all with the same vitamin E content, from 2-weeks before conception, throughout pregnancy and lactation. After the breast-feeding period (28 days after birth), offspring coming from mothers fed different oil types and sources were weaned with the same diet for 50 days. The results showed that the dietary fat content in the maternal diet induced dys-regulation of fatty acid desaturase 1 gene (*Fads1*) and *Fads2* in offspring aorta. In addition, there was an altered epigenetic regulation of the *Fads2* promoter at the CpG-394 region in both sex groups that correlated with the dietary lipid content, regardless the dietary sources tested. This study demonstrated that in rats maternal nutritional programming can regulate epigenetic mechanisms such as promoter methylation of *Fads2* in cardiovascular tissues, affecting the *de novo* synthesis of LC-PUFA and that the proportions of these fatty acids in offspring hearth can be regulated by early nutritional interventions through the mother's diet (Kelsall *et al.*, 2012). In a following study (Hoile *et al.*, 2013) feeding

from 14 days preconception until weaning with diets containing 3.5, 7 or 21% lipids high in saturated fatty acids (from butter) or n-3 LC-PUFA (from FO), maternal nutrition during the pregnancy and lactation regulated *Fads2* expression also in the liver (a major site of fatty acid synthesis). Increase in dietary lipid contents up to 21% in mother diets reduced ARA (20:4n-6) and DHA (22:6n-3) contents in liver lipids, even when FO was used as the lipid source. Moreover, a negative correlation was found between *Fads2* expression and the methylation of *Fads2* promoter at the CpG sites –394, –84 and – 76 (Hoile *et al.*, 2013). Indeed, there was a correlation between the ARA (20:4n-6) and DHA (22:6n-3) concentrations in the liver and the *Fads2* expression levels in the same tissue indicating higher biosynthesis of n-3 LC-PUFA (Hoile *et al.*, 2013). However, establishing the effects of maternal nutrition on the offspring capacity to confront adverse conditions (such as limitations in n-3 LC-PUFA) was not in the scope of that study (Hoile *et al.*, 2013).

In comparison to what occurs in mammals, in fish, research about the potential nutritional programming effect of lipids and, particularly, fatty acids through parental nutrition have received scarce attention. Pioneer studies, feeding gilthead sea bream and long snout seahorse (*Hippocampus reidi*) broodstock with diets differing in their fatty acid profiles, demonstrated the long-term effects resulting from parental epigenetic control of embryogenesis, as well as the potential of nutritional conditioning during early larval stages (Izquierdo, 2013; Otero-Ferrer *et al.*, 2013). Thus, to define the dietary factors that influence the epigenetic profile, the most sensitive time-windows and the length of intervention three types of programs were conducted in gilthead sea bream by exposing embryos, larvae or post larvae to diets containing different FO/VO ratios (Izquierdo, 2013). The study demonstrated that nutritional programming through broodstock feeding (namely exposure to different fatty acids profiles during early embryonic development) was more effective than programming during larval stages in gilthead sea bream. Therefore, after rearing the offspring with the same commercial diets and feeding protocols, those juveniles obtained from broodstock fed VO showed a better ability to use low FO diets (Izquierdo, 2013; Izquierdo, 2013), through the modulation of lipid metabolism (Turkmen and Izquierdo, 2014). However, the potential long-term effects of nutritional programming through broodstock diets, their combination with

stimulus conducted later in offspring life, the potential interactions with the broodstock genetic background, the responses to either low FO diets or low FM and FO diets or the potential epigenetic mechanisms implied had not been yet addressed.

Studies in long snout seahorse allowed to compare the potential nutritional programming effect of paternal or maternal nutrition, showing that seahorses are a valuable and effective epigenetic model (Otero-Ferrer *et al.*, 2013). In these studies, either mysids containing either high or low levels of LC-PUFA were fed separately to males or females of 16 seahorse breeding pairs prior to mating for 5 months. As expected, offspring obtained from high LC-PUFA fed females were larger than those from low n-3 LC-PUFA fed females (Otero-Ferrer *et al.*, 2013). Interestingly, these studies also demonstrated that the nutritional status of the male is determinant of the morphology and feeding habits of the offspring, independently of the mother's diet. Thus, a low nutritional quality in male diets before conception and pregnancy, produced abnormally large and heavy offspring with low survival prospects, even if females are given a high-quality diet (Otero-Ferrer *et al.*, 2016). Moreover, offspring fatty acid profiles were affected by broodstock dietary treatment, including male diet. Thus, when females were given the low LC-PUFA diet, both ARA (20:4n-6) and EPA (22:6n-3) were lower in offspring of males fed low LC-PUFA mysids than in those from males fed high LC-PUFA (Otero-Ferrer *et al.*, 2016). These studies point out the important contribution of parental feeding, both females and males, for offspring phenotypes and lipid metabolism.

In red drum (*Sciaenops ocellatus*), broodstock were fed with different mixtures of ingredients such as shrimp, squid, mackerel, beef liver, Spanish sardines or capsules containing an ARA (20:4n-6) supplement in 8 different combinations (Perez and Fuiman, 2015) during spawning and obtained different contents of ARA (20:4n-6) and DHA (22:6n-3) in the eggs (1·8–5·1% of total fatty acids for ARA (20:4n-3) and 7·3–24·4% for DHA(22:6n-3))(Perez and Fuiman, 2015). Moreover, despite the larvae obtained from those broodstock were fed with different profiles of EFA, the DHA (22:6n-3) contents in the egg markedly affected larval escaping response and survival, even if the larval diet was high in DHA (22:6n-3) (Perez and Fuiman, 2015). However, the insufficient

information about the broodstock diet's biochemical composition made difficult to define if this modulation was related to fatty acid composition and/or lipid content of the diets. In a series of studies analysing the fatty acid profiles of red drum eggs and larvae fed different fatty acid profiles (Fuiman and Perez, 2015). The results clearly showed that both eggs fatty acid profiles and dietary fatty acids affect the DHA (22:6n-3) contents of the larvae (Fuiman and Perez, 2015).

In Senegalese sole, fortification of the broodstock diet with lipids, vitamins and LC-PUFA resulted in a down-regulation of the expression of genes from certain enzymes such as fatty acid elongase 5 (*elovl5*) and Δ4-desaturase (*Δ4fad*), involved in LC-PUFA biosynthesis, in the larvae produced (Morais *et al.*, 2014). This study provided clues that broodstock nutrition can have effects on the nutritional programming of lipid metabolism through the broodstock nutrition (Morais *et al.*, 2014). Moreover, higher levels of DHA and EPA did not lead a better survival, conversely resulted in diminishing effects in *elovl5* and *Δ4fad* gene expression in the offspring (Morais *et al.*, 2014).

By the primary promising results observed in the literature -in addition to the results obtained in this thesis -a broader interest in research nutritional programming of lipid metabolism through early nutritional interventions has grown in aquaculture. In more recent studies, in a principal species for aquaculture in the world (4% of total aquaculture production (FAO, 2018)), Atlantic salmon, the effects of nutritional programming by early interventions using FO or VO based diet have been studied (Clarkson *et al.*, 2017; Vera *et al.*, 2017). In these studies, first exogenous feeding was considered as a nutritional programming window when Atlantic salmon fry were fed either a marine or a vegetable-based diet for 3 weeks. Then fish were kept in the same environmental and diet conditions and at 18-months of age they were challenged with a vegetable-based diet (Clarkson *et al.*, 2017). In this study, 24% higher growth rate was found in salmon that had been fed a vegetable-based diet during the early developmental than those fed a marine-based diet (Clarkson *et al.*, 2017). Later studies showed that nutritional interventions in the larval stage with a vegetable-based diet caused a differential expression of genes involved in carbohydrate, amino acid and lipid metabolism (28%

difference), signalling (15%), and a lesser extent (7%) immune system, endocrine system and translation (Vera *et al.*, 2017). Particularly, genes involved in fatty acid desaturation (*fads2* (1.2 fold), *scd1* (1.6 fold)), elongation of very long-chain fatty acids (*elovl4* (1.4 fold), *elovl5* (1.6 fold), *elovl6* (2.3 fold)) were up-regulated in comparison to fish that received a marine-based diet during the fry stage for 3 weeks (Vera *et al.*, 2017). The same study also showed that there was a small but significant change in the global % methylation ratios of 5mdCytosine of total cytosines in triploid individuals but not in diploids which may indicate that epigenetic mechanisms are also involved in the regulation of the differentially expressed genes in liver (Vera *et al.*, 2017). Some of these studies and their key results were summarized in Table 1-1.

Table 1-1 I Summary of nutritional programming by n-3 LC-PUFA diets in early developmental stages from the published results obtained in different fish species and the experiments conducted in this thesis

	Nutritional programming		Maintenance	Challenge test		Results	
Specie	Developmental window (Time – duration)	Diet	Diet	Time (Duration)	Diet		
Gilthead sea bream (*Sparus aurata*)	Larval development (Early, 3 dah to 18 dah)	-Rotifer enrichments 14.1%n-3 LC-PUFA + 2.6%ALA 10.1% n-3 LC-PUFA + 16.5%ALA	Commercial diets	8-months-old (1 month)	100% FM FO 5% FM 3%FO	VO inclusion in the broodstock diets caused lower growth at juvenile stage after the challenge test for all the diets tested.	Unpublished data
Senegalese sole (*Solea senegalensis*)	Broodstock (2 years until spawning)	-Control (FM&FO) -Supplemented diet (FM&FO) + vitamins + n-3 LC-PUFA	Commercial diets	Direct observations	Commercial-like diet	Control (lower n-3 LC-PUFA) fed eggs and newly hatched larvae showed higher transcript levels of *elovl5* and *Δ4fad*. No significant effect on the juvenile growth at 67 dah if fed with only commercial diets.	(Morais *et al.*, 2014)
Atlantic salmon (*Salmo solar*)	Larval development First exogenous feeding – 3 weeks	-Marine-based control (100% FM&FO) -Vegetable-based (80%FM – 4%FO)	Commercial diets	18-weeks-age (3 weeks)	100% FO 100% VO -Rapeseed oil	24% higher growth rate and 23% better feed efficiency in vegetable-based stimuli group if fed with 100% VO diet.	(Clarkson *et al.*, 2017)
"	"	"	"	"	"	Nutritional programming with vegetable-based diets induced up-regulation in genes involved in phosphorylation, pyruvate metabolism, TCA cycle, glycolysis and fatty acid metabolism in liver.	(Vera *et al.*, 2017)
Long snout seahorse (*Hippocampus reidi*)	Broodstock	Commercial or willd-caught preys before mating	Commercial diets	Direct observations	-	Manipulating the nutritional status of the male seahorse has important effects on subsequent embryonic development and that these effects are independent of the maternal diet. preconception nutritional status of Females exerts different, but equally important, effects on embryonic development that are unrelated to the male's diet.	(Otero-Ferrer *et al.*, 2016)
Gilthead sea bream (*Sparus aurata*)	Larval development (Late, 16 dah to 26 dah)	-Marine based diet lipid source krill oil -25% substitution by LO + (SL) -50% substitution by LO + SL -75% substitution by LO + SL -100% substitution by LO + SL	-	-	-	Fish fed low n-3 LC-PUFA at 16 dah constrained the feasibility of nutritional programming needs to be further investigated. Up-regulation of *fads2* in fishes fed 50% and 75% substitution levels.	(Turkmen *et al.*, 2017)

FO: Fish oil, VO: Vegetable oil, LO: Linseed oil, SL: Soy bean lecithin

Table 1-1 Continued| Summary of nutritional programming by n-3 LC-PUFA diets in early developmental stages from the published results obtained in different fish species and the experiments conducted in this thesis

	Nutritional programming		**Maintenance**	**Challenge test**		**Results**
Specie	Developmental window (Time – duration)	Diet	Diet	Time (Duration)	Diet	
Gilthead sea bream (*Sparus aurata*)	Broodstock diet (1 month before egg collection)	-100% FO -40%FO – 60%LO -20%FO – 80%LO -100%LO	Commercial diets	4-months-old	-100% FO -20%FO – 80%LO -100%LO	**Chapter 3**
	"	"	"	4-months-old	100% FM FO 5% FM 3%FO	**Chapter 4**
	"	"	Commercial diets & Reminder diet (5% FM 3%FO) at 4-months-old	16-months-old	5% FM 3%FO	**Chapter 5**
	Broodstock diet (1 month before egg collection) + Broodstock selection	-100% FO -30%FO – 60%LO	Commercial diets	6-months-old	5% FM 3%FO	**Chapter 6 & Chapter 7**

FO: Fish oil, VO: Vegetable oil, LO: Linseed oil, SL: Soy bean lecithin

In order to further investigate on the nutritional programming effects of fatty acid profiles in parental diets on the ability of the progeny to use diets low in FM and FO, gilthead sea bream was selected as a model species to continue with the pioneer research conducted in this species.

Overall, these studies show that there are differential responses to the nutritional interventions during the early developmental stages (Vagner *et al.*, 2007; Vagner *et al.*, 2009; Izquierdo, 2013; Izquierdo, 2013; Otero-Ferrer *et al.*, 2013; Morais *et al.*, 2014; Turkmen and Izquierdo, 2014; Fuiman and Perez, 2015; Perez and Fuiman, 2015; Otero-Ferrer *et al.*, 2016; Clarkson *et al.*, 2017; Vera *et al.*, 2017). Although the research to date in this topic is very limited, in addition to the environmental factors confronted during the early developmental stages, the response seems to be related with differences in species own physiology, nutritional intervention type, duration and time of the developmental windows. Also, it must be taken into account that different fish species have diverse survival strategies. For instance, in general speaking marine fish species produce higher number of eggs, that are smaller, hatched larvae has a very limited digestion capacity since the gastric glands and acidic digestion do no present until the metamorphosis (Govoni *et al.*, 1986). In the light of the previous results and gilthead sea bream being a multi-batch spawner whose oligolectic eggs largely depend on their continuous intake of nutrients during reproduction, nutritional programming of lipid metabolism using VO based diets in gilthead sea bream, seems more promising if nutritional intervention is applied through the broodstock diets during the spawning period. In addition, although there are several studies that demonstrated the parental effects on future health and metabolism of the progeny, the mechanisms of the epigenetic inheritance are still not very clearly shown. Recently, studies in another model animal *Drosophila*, showed histone methylation modulated by H3K27me3, is associated with repressing of gene expression, mothers pass epigenetic marks to the offspring and these epigenetic marks are crucial for the embryogenesis by activation of expression of different transcriptional genes (Zenk *et al.*, 2017). Thus, broodstock nutrition is an interesting developmental window both for possible inheritance programming by the

parents as well as supplying nutritional interventions in very early stages of embryonic development.

1.6 Importance of gilthead sea bream aquaculture

Gilthead sea bream is a major species for the European aquaculture. Although sea bream production is more intense in the Mediterranean Sea, production also exists in Atlantic and Indian oceans as well as in inland waters of Africa (FAO, 2018). The aquaculture production of the gilthead sea bream accounts nearly 190000 tones in 2016 (FAO, 2018)(Figure 1-6). From 2012 to 2016, gilthead sea bream aquaculture production has kept a steady growth (FAO, 2018). This species, together with the European sea bass, is one of the major marine species in the Mediterranean aquaculture. According to the most recent data (2016), Turkey is the major producer with 31% of the total aquaculture production, followed by Greece and Egypt with 26% and 14% of the total production (FAO, 2018)(Figure 1-6).

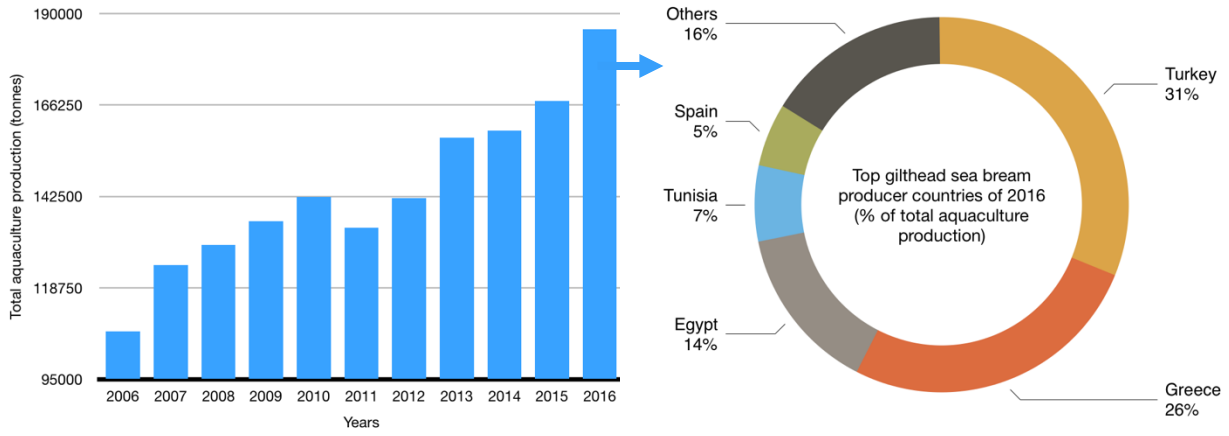

Figure 1-6 | Gilthead sea bream production over the last ten years of the available data from FAO 2018, pie chart shows % contribution of total production of 2016.

1.7 Reproduction properties of gilthead sea bream

The life-cycle of gilthead sea bream is presented in Figure 1-7. The gilthead sea bream is a protandrous hermaphrodite that behaves as a functional male at least during the first two years of life. At the first year of its life the gonad undergoes juvenile hermaphroditism, with the young bisexual gonad developing toward an ovary but then

subsequently toward a testis. Ultimately, the entire year class functions as males during their first reproductive maturity as one-year-old fish. Later at the second-year ratio of sex change to male to females increases being much higher at the third year (Kadmon *et al.*, 1985).

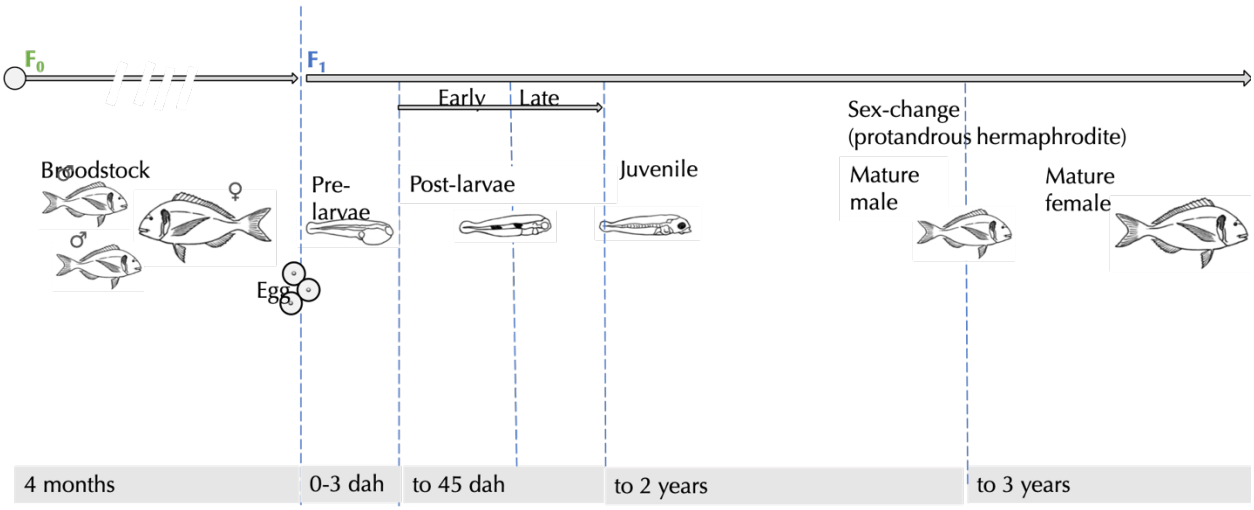

Figure 1-7 | The life-cycle of gilthead sea bream

Gilthead sea bream has a large spawning season and may spawn daily for a period of 3 to 5 months, producing up to 2 million eggs per kg of body weight of female brood fish (Fernández-Palacios, 2005).

One of the most important aspects of the gilthead sea bream spawning period is feeding (Izquierdo *et al.*, 2001), as having asynchronous ovaries require a continuous nutrient supply to develop the viteliogenetic ova. In gilthead sea bream, the egg yolk nutrient composition is directly related with the continuous feed intake during the reproduction period. Therefore, the quality of the eggs is directly affected by the n-3 LC-PUFA levels of the broodstock diet (Fernández-Palacios, 2005). Moreover, the nutrient composition of the eggs can be directly modified by the broodstock diet only in 3 weeks of feeding (Fernández-Palacios, 2005). However, due to the high risk of oxidation of n-3 LC-PUFA, high inclusion levels of 2.2% of the diets, may reduce the spawning quality parameters as well as larval survival by 10% (Fernández-Palacios, 2005). However, inclusion of sufficient levels of vitamin E can prevent the adverse effects of high n-3 LC-PUFA levels and improve the spawning quality in gilthead sea bream (Fernández-

Palacios, 2005). Due this property of this species, broodstock nutrition gives a special possibility to influence the nutrients supplied through the egg yolk composition in very early stages of the development.

1.8 Objectives

Being feed cost one of the main maintenance expenses in aquaculture, supply of economically sustainable feeds is utmost important. Traditionally, FM and FO were basic protein and lipid sources in finfish diets but constrains in their availability and increasing in higher prices have seriously limited their use in aquafeeds. This has forced the feed production industry to search for more sustainable and economic alternatives. While marine originated oils are rich in n-3 LC-PUFA (essential for marine fish to promote growth and health), VO are lacking these fatty acids but are rich in n-6 and n-9 fatty acids of 18C. Generally speaking, marine fish have a strict requirement for PUFA, including ARA (20:4n-6), EPA (20:5n-3) and DHA (22:6n-3) (Sargent *et al.*, 1999). Consequently, marine fish seem to have a lower ability to utilize terrestrial oils due their high n-3 LC-PUFA requirements and the insufficient activity of enzymes synthetizing n-3 LC-PUFA from the fatty acid precursors found in VO. Thus, substitution of FO with VO seems more challenging than in other species that are better equipped to utilize dietary lipids more efficiently. The concept of early-programming raises the interesting possibility of directing specific metabolic pathways or functions in juvenile fish. For example, nutritional programming may improve the use of substitutes to FM and FO, and hence to promote sustainability in aquaculture by maximizing the capacity of lipid utilization in fish. Thus, the main objectives of this thesis were to:

1. To investigate the potential value of fatty acids as modulators of early nutritional programming and determine the effect of parental feeding with VO diets on offspring performance along life spam, focusing on egg quality, larval and juvenile growth. Two different long-term trials were conducted and presented in Chapter 3 and Chapter 4.

2. To establish the effect of parental feeding with VO diets on offspring lipid metabolism, analyzing proximate and fatty acid composition and expression of selected genes in eggs, larvae and juveniles (Chapter 3 and Chapter 4).
3. To analyze the effect of parental feeding with VO diets on expression of selected genes related to health and stress resistance of offspring (Chapter 3).
4. To study the effect of parental feeding with VO diets on offspring juveniles ability to use high VO diets. For that purpose, juveniles offspring were challenged with diets low in FO in Chapter 3.
5. To determine the effect of parental feeding with VO diets on offspring ability to use diets high in VO and VM. This goal was addressed by two feeding trials with offspring juveniles fed a low FO and low FM diet (Chapter 4 and Chapter 6).
6. To find out if there is a persistence of the nutritional programming effects through parental feeding in offspring in the beginning of first gonad development and if this affects fillet quality for the consumer. To address this objective, the effects of both broodstock feeding and an early nutritional challenge in juvenile stage on growth, chemical and fatty acid composition of muscle and liver, and expression of selected genes in the liver were investigated in Chapter 5.
7. To investigate if there is a combine effect of the nutritional conditioning of broodstock and the nutritional challenge of juveniles in the offspring ability to use diets high in VO and VM (Chapter 5).
8. To determine the potential interaction between selection of fish with a higher expression of *fads2* and nutritional programing through broodstock nutrition. For that purpose, sea bream broodstock was either selected for high or for low *fads2* response and fed with diets either high in FO or in VO and their offspring performance and utilization of a low FM and FO diet were investigated in Chapter 6.
9. To better understand the potential epigenetic mechanisms implied on nutritional programming of gilthead seabream through broodstock conditioning. For that purpose, in Chapter 7 the potential DNA methylation of promotors of specific genes were studied.

Chapter 2

GENERAL MATERIALS AND METHODS

2.1 General design and experimental animals

In this part a general experimental design will be mentioned. Afterwards, the specific characteristics of each trial will be detailed in each chapter. All the experiments mentioned in the thesis follows a similar theoretical experimental design. Briefly, a nutritional stimulus is applied to the broodstock fish during the spawning period, at least for 1 month before seeding the eggs for rearing the progeny, in order to modify the egg fatty acid profiles. Those eggs obtained from differently fed broodstock groups were produced under the same conditions and fed commercial feeds and feeding protocols until the desired juvenile stage. Afterwards, juveniles were nutritionally challenged with low FM FO diets in order to test the performance of the offspring during the juvenile stage. In all studies, the gilthead sea bream used were produced and reared in different facilities of the EcoAqua Institute (Telde, Canary Islands, Spain). A general schematic presentation of the experimental design showed in Figure 2-1.

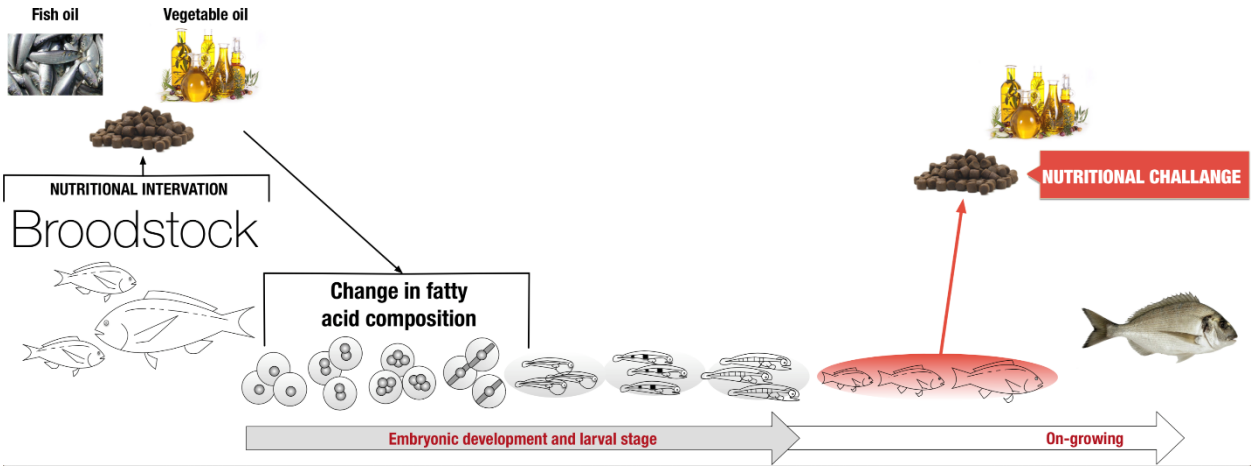

Figure 2-1 I General experimental design of the experiments

2.1.1 Broodstock

Gilthead sea bream broodstock used in these studies were produced at EcoAqua (Telde, Canary Islands, Spain) facilities in captivity. Generally, 2 to 4 years-old brood fish were used for each experiment of this thesis.

2.1.1.1 Preparation for the spawning season

Prior to the spawning season, broodfish were randomly selected from a larger group of fish held together in 10000 L capacity tanks. All the broodfish held in this tank were pit-tagged. Before the placement of the broodfish into the experimental tanks, fish were identified by their pit-tag numbers, weighted and sized for total length. Although the sex determination is easy by size differences of male and female individuals, the sex determination was made by a gentle massage on the abdominal cavity, using small pressure by fingers to identify mature males. These recorded weights before the spawning season were used as reference for each individual to calculate the necessary amount of feed.

2.1.1.2 Phenotypic selection of broodfish

In chapters 6[t] and 7, brood fish were selected according to their response in *fads2* expression 3 months before the spawning season. Before the selection, broodfish were fed a low FM and FO diet, low in LC-PUFA and high in 18C fatty acids (please see 2.2 Diets section) to trigger LC-PUFA biosynthesis and the expression of related genes. To keep broodfish alive and avoid excessive stress, blood was taken from anesthetized gilthead sea bream broodfish. The sampling procedure was given in 2.3 Sampling section of this chapter.

2.1.1.3 Spawning, spawning quality assessments and egg collection

Spontaneously spawned eggs obtained from each experimental broodstock were collected five times per week, during the whole experimental periods. Spawning and egg quality were determined by quantifying the total number of eggs and larvae produced, and the proportion of fertilized, viable and hatched out eggs were calculated (Fernández-Palacios *et al.*, 2011). Briefly, collected eggs were placed in 5 L beakers provided with

aeration, from where 5 randomized 5 mL samples were place in a Bogorov chamber, counted and observed under binocular microscope to calculate: Total amount of eggs, percentage of fertilized eggs, determined by the morphological characteristics of the eggs (Fernández-Palacios *et al.*, 2011); and egg viability rate, determined as the percentage of morphologically normal eggs at the morula stage and described as transparent, perfectly spherical with clear, symmetrical early cleavages (Fernández-Palacios, 2005). After that, another 5 randomized 5 mL samples were individually place in a Bogorov chamber. Eggs were counted and transferred to five 250 mL crystal beakers filled with sterilized seawater. All the beakers were maintained in a continuous seawater bath to maintain constant temperature. From these samples two different parameters were calculated: hatching rate and larval survival at 3 dah (Fernández-Palacios *et al.*, 1995). With this percentages the total numbers of fertilized, viable and hatched eggs and larvae produced per kg female were calculated. Broodstock fish were fed with experimental diets for 1 month at least before the egg collection. Since previously studied, 1 month was sufficient to influence the fatty acid profiles of gilthead sea bream eggs through the diets. After the experimental period, eggs were collected and always stocked with 100 egg/L ratio to the corresponding tanks and each experimental group kept separately.

2.1.2 Larvae production

Larvae were produced using the protocols established in EcoAqua Institute facilities. Fertilized eggs were stocked into separated mass production tanks (2000 L) at a density of 100 eggs/mL and larvae were produced under the same commercial protocol, regardless the respective parental diet. In brief, sand filtered, and UV sterilised seawater flow was progressively increased from 10 to 40% per h until 46 dah; water was continuously aerated (125 mL/min); average water temperature and pH along the trials were recorded; a 1500–3500 lx 12 h single central light (Mod TLD 36 W/54, Philips, France) photoperiod was provided and living phytoplankton (*Nannochloropsis sp.*) (250 ± 100 × 10^3 cells/mL) was added to the rearing tanks. Regarding feeding, from 3–32 dah, larvae were fed twice a day with rotifers (*Brachionus plicatilis*) at 5–10 rotifers/mL

enriched with commercial emulsions (ORI-GREEN, Skretting, Norway); from 15–17 dah, *Artemia sp*. enriched with commercial emulsions (ORI-GREEN, Skretting, Norway) were added three times a day; and from 20 dah, larvae were fed commercial diets (Gemma Micro, Skretting, France).

2.1.3 Juvenile production and nutritional challenge of juveniles

After the larval stage, obtained larvae were transferred to 5000 L pre-weaning tanks. Fish were fed with corresponding commercial diets of increased diameter depending on fish size. Fish were regularly sized and graded to avoid cannibalism and to select the appropriate feed size. Then fish were reared to 2 g and were transferred to 1000 L flow-through tanks in triplicates. Once the offspring obtained from different broodstock groups reached desired size (3 g or 6 g), fish were randomly selected and moved to the experimental tanks for the nutritional challenge. All the fish were fed *ad libitum* for the nutritional challenge tests (2.2.3 Juvenile stages and nutritional challenge section). For the feeding, 50 g of feed were weighted before the first feeding and after the last one feeds were weighted again to calculate the feed intake for each tank daily. In general, experiments were conducted until fish duplicated the initial size. All the calculations for the growth parameters were explained in 2.3.1.4 growth parameters estimation section.

2.2 Diets

2.2.1 Broodstock

2.2.1.1 Acclimation periods

For the broodstock experiments, during the acclimation period and until similar spawning parameters were observed among groups, a commercial diet especially formulated for broodstock was used (VitalisRepro, Skretting, Norway). The feeding ratio was 1% of the total mass of each experimental tank.

2.2.1.2 Nutritional programming

2.2.1.2.1 Experiment one, effects of parental diets with decreasing levels of n-3 LC-PUFA on selected PUFA metabolism and immune system related genes in gilthead seabream offspring focusing on the larval and juvenile stage

In the first experiment, 4 different diets with increasing substitution of FO by LO tested. The formulation of the diets was presented in Table 2-1. Broodstock groups were fed with these four diets and effects on the progenies focusing on the stress response at the larval stages as well as growth performance of the progenies were tested.

Table 2-1 | Main ingredients, proximate composition and energy contents in the practical diets used for the nutritional programming of gilthead seabream broodstock fed different substitution levels of fish oil by vegetable oils

Main ingredients (%)	**FO**	**60LO**	**80LO**	**100LO**
Marine meals[1]	50.0	50.0	50.0	50.0
Sunflower cake	13.2	13.2	13.2	13.2
Soya cake[2]	10.0	10.0	10.0	10.0
Wheat gluten[3]	9.9	9.9	9.9	9.9
Corn gluten[4]	7.0	7.0	7.0	7.0
Fish oil[5]	8.0	3.2	1.6	-
Linseed oil[6]	-	4.8	6.4	8.0
Vitamin and mineral premix[7]	1.0	1.0	1.0	1.0
Proximate composition (%)				
Moisture	7.73	8.09	7.98	8.08
Crude protein	52.92	53.63	54.50	53.29
Crude lipids	19.51	19.09	19.13	19.48
Ash	8.08	8.37	8.25	8.00
Digestible energy (MJ/kg)	21.20	21.20	21.20	21.20

1 Contains Fish meal NA LT 70, Fish meal SA 68, Feed Service Bremen, Germany.
2 48 Hi Pro Solvent Extra. Svane Shipping, Denmark
3 Hedegaard, Denmark
4 Cargill, Netherlands
5 South American fish oil, LDN Fish Oil, Denmark
6 Ch. Daudruy, France
7 Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany)

2.2.1.2.2 Experiment two and three, validation of nutritional programming in gilthead sea bream through broodstock diets and defining the optimal vegetable oil substitution levels

Same diets were used as the first experiment Table 2-1. Broodstock groups were fed with these four diets and effects on the progenies were tested in juvenile stage.

2.2.1.2.3 Experiment four and five, combined effects of nutritional programming with 18C rich fatty acid diet and genetic selection of the broodstock to improve utilization of vegetable meal and oil diets

In these experiments, firstly a genetic selection of the broodstock with high or low *fads2* expression levels were made (detailed in 2.1.1.2.) In order to make the genetic selection, the broodstock was fed with a low FM and FO diet in order to trigger the *fads2* expression. For this experiment a diet with low n-3 LC-PUFA were used. Diet formulation was supplied in Table 2-2.

Table 2-2 l Main ingredients*, energy, protein and % total fatty acids contents of diets used in conditioning test

Main Ingredients	%	Proximate Composition	(% dry matter)
Fish meal SA[1] 68 super prime	5.0	Crude lipids	21.7
Fish meal alternative protein sources[2]	54.5	Crude protein	45.1
Rapeseed meal cake	11.3	Moisture	9.0
Wheat	6.9	Ash	5.4
Fish oil SA[1]	3.0		
Vegetable oil mix[3]	13.0	**Gross Energy (MJ/kg, as is)**	**22.5**

% total fatty acids			
16:1n-7	2.1	20:5n-3	2.5
18:2n-6	20.3	22:1n-11	0.1
18:3n-3	11.8	22:6n-3	1.7

* Please see Torrecillas *et al.*, (2017) diet code 5FM/3FO for the complete list of feed ingredients.
1. South American, Superprime (Feed Service, Bremen, Germany).
2. Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
3. Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

After this conditioning period to identify the *fads2* expression in blood, fish were fed with 2 experimental diets for the nutritional programming for 1 months (Table 2-3).

Table 2-3 I Formulation, main ingredients and biochemical composition of the nutritional programming diets supplied 1 month before egg collection for larval rearing

Raw material (%)	100% FO (F)	30% FO-70% VO (V)
Meals from marine sources[1]	50.0	50.0
Sunflower cake	13.2	13.2
Soya cake[2]	10.0	10.0
Fish oil[3]	8.0	2.4
Linseed oil[4]	-	5.6
Wheat	9.9	9.9
Corn gluten 60	7.0	7.0
Drying / wetting	0.9	0.9
Vitamin & mineral premix[5]	1.0	1.0
Vitamin E powder (50%)	0.1	0.1
Biochemical composition (% of dry matter)		
Moisture	9.1	8.8
Protein (crude)	56.3	56.1
Lipids (crude)	17.2	17.1
Ash	8.6	8.5
Energy - gross (MJ kg^{-1})	21.2	21.2
Fatty acids (% of total fatty acids)		
16:1n-7	7.1	4.3
18:2n-6	5.6	9.9
18:3n-3	0.9	16.3
20:1n-7	12.4	6.8
20:4n-6	0.4	0.3
20:5n-3	6.3	4.8
22:1n-11	15.7	8.6
22:6n-3	7.1	6.0

[1] Contains Fish meal NA LT 70, Fish meal SA 68, Feed Service Bremen, Germany.
[2] 48 Hi Pro Solvent Extra. Svane Shipping, Denmark
[3] South American fish oil, LDN Fish Oil, Denmark
[4] Ch. Daudruy, France
[5] Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany)

2.2.2 Larval stages

Protocol for the larval rearing was comparable to those commercial farm protocols. For all the experiments larval rearing the feeds and the feeding protocols were similar. Briefly, eggs were collected and distributed to 500 L tanks for mass production at a density of 100 eggs/L from each broodstock groups. Water renewal in the tanks were progressively increased from 10 to 40% per h until 46 dah. Water was

continuously aerated (125 mL/min). Larvae were reared under natural photoperiod and living phytoplankton (*Nannochloropsis sp.*) (250 ± 100 x 10^3 cells/mL) was added to the rearing tanks. From 3-32 dah, larvae were fed twice a day with rotifer (*Brachionus plicatilis*) at 10 rotifers/mL enriched with commercial emulsions (ORI-GREEN, Skretting, Norway) and from 15-17 dah, *Artemia sp.* enriched with same emulsions were added 3 times a day. From 20 dah, larvae were fed commercial diets (Gemma Micro, Skretting, France). Water was continuously aerated (125 mL/min). Average water temperature and pH along the trial were checked. Water quality was daily monitored by dissolved O_2 and pH. At 46 dah, the whole population were transferred to 10,000 L tanks.

2.2.3 Juvenile stages and nutritional challenge

After the progeny were reached to the desired size, juveniles were challenged with diets low in fish meal and oil to test the effects of the broodstock diet on the progeny. Diets were formulated according to the aims of the juvenile nutritional challenge.

2.2.3.1.1 Experiment one, effects of parental diets with decreasing levels of n-3 LC-PUFA juvenile stage when fed the same diets as their parents

In experiment 1, progeny obtained from broodstock fed with four different diets (Table 2-1), were nutritionally challenged using similar diets as the broodstock feeds. These diets are designed to test the possible effects of nutritional programming of the offspring and feed utilization if they feed with the similar diets as their parents expect the lowest linseed oil 60LO diet (Table 2-1). The formulations of the challenge diets were shown in Table 2-4. Juveniles from each group were daily fed until apparent satiation for 60 days, three times a day at 09:00, 13:00 and 17:00 hours. The feed was supplied in small portions (< 5-6 pellets at a time) to ensure that all feed was eaten. After each feeding, uneaten feed was collected, kept in aluminium oven trays, dried overnight at 105 ºC and weighted to calculate feed intake. Feed consumption was recorded.

Table 2-4 l Main ingredients, proximate composition and energy contents in the practical diets used for the nutritional programming of gilthead seabream broodstock fed different substitution levels of fish oil by vegetable oils

Main ingredients (%)	**F0**	**80L0**	**100L0**
Marine meals[1]	50.0	50.0	50.0
Sunflower cake	13.2	13.2	13.2
Soya cake[2]	10.0	10.0	10.0
Wheat gluten[3]	9.9	9.9	9.9
Corn gluten[4]	7.0	7.0	7.0
Fish oil[5]	*8.0*	*1.6*	-
Linseed oil[6]	-	*6.4*	*8.0*
Vitamin and mineral premix[7]	1.0	1.0	1.0
Proximate composition (%)			
Moisture	7.73	7.98	8.08
Crude protein	52.92	54.50	53.29
Crude lipids	19.51	19.13	19.48
Ash	8.08	8.25	8.00
Digestible energy (MJ/kg)	21.2	21.2	21.2

[1] Contains Fish meal NA LT 70, Fish meal SA 68, Feed Service Bremen, Germany.
[2] 48 Hi Pro Solvent Extra. Svane Shipping, Denmark
[3] Hedegaard, Denmark
[4] Cargill, Netherlands
[5] South American fish oil, LDN Fish Oil, Denmark
[6] Ch. Daudruy, France
[7] Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany)

2.2.3.1.2 Experiment two, testing the possible nutritional programming effect through the broodstock nutrition in 3-months-old juveniles those are obtained from parents with increasing levels of FO substitution by LO

In experiment 2, the progeny obtained from broodstock fed with different diets (Table 2-1), was nutritionally challenged using very low FM 5% and FO 3% (VOVM diet) and 100% FM / FO (FOFM) control diet. These diets are designed to test the possible effects of nutritional programming of the offspring and feed utilization. 100% FM / FO diet was used as a control diet to test the general growth performance of the experimental groups. The formulations of the challenge diets were shown in Table 2-5.

Diets were formulated and produced by Biomar company, Denmark and they were isoenergetic, one being high in FO (15.30%) and FM (58%) (FOFM diet), and the other extremely low in FO (3%) and FM (5%) (VOVM diet)(Table 2-5). Thus, the FOFM diet had higher levels of ARA, EPA and DHA, whereas the VOVM diet was higher in oleic acid(18:1n-9), LA (18:2n-6), and ALA (18:3n-3) (Table 2-5). Juveniles from each broodstock were daily four times a day (08:00, 11:00, 14:00 and 17:00h) fed until apparent satiation with one of the two different diets in triplicates for 30 days. Feed consumption was daily recorded.

Table 2-5 I Main ingredients, energy, protein and some fatty acids (% total fatty acids) of diets for the nutritional challenge of gilthead seabream juveniles obtained from broodstock fed different FO and LO ratios

Raw material (%)	**FOFM (F)**	**VOVM (V)**
Fishmeal SA 68 Superprime [1]	58.00	5.00
Alternative protein sources [2]	-	54.50
Rapeseed cake	10.00	11.30
Wheat	15.88	6.89
Fish oil SA*	15.30	3.00
Alternative lipid sources**	-	13.00
Fishmeal SA 68 Superprime [1]	58.00	5.00
Alternative protein sources [2]	-	54.50
Biochemical composition (% of dry matter)		
Moisture	10.20	10.96
Protein (crude)	47.80	46.67
Lipids (crude)	21.23	22.45
Ash	10.20	10.96
Energy - gross (MJ kg^{-1})	18.96	18.89
Fatty acids (% of total fatty acids)		
18:1n-9	0.09	0.05
18:2n-6	3.55	19.57
18:3n-3	1.38	11.47
20:4n-6	0.99	0.23
20:5n-3	15.13	3.27
22:6n-3	11.12	2.40

1. South American, Superprime (Feed Service, Bremen, Germany).
2. Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
**Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

2.2.3.1.3 Experiment three, testing the persistence of nutritional programming on growth, lipid metabolism and utilisation in later developmental stages the offspring are 16-months-old

The juveniles obtained from different broodstock groups and nutritionally challenged at 4-months-old kept separately in different tanks in triplicates and all the groups were fed with commercial diets until they reach 16-months-old. This experiment was designed to test the long-term effects of different broodstock diets on the progeny. In addition, nutritional challenge test at 4-months-old with VM-VO diet (Table 2-5), considered as a reminder diet while FM-FO diet as the control diet. At 4 months old fish were again challenged with a very low FM and FO diet (Table 2-6). Juveniles from each group were daily fed until apparent satiation for 60 days, three times a day at 09:00, 13:00 and 17:00 hours. The feed was supplied in small portions (< 5-6 pellets at a time) to ensure that all feed was eaten. After each feeding, uneaten feed was collected, kept in aluminium oven trays, dried overnight at 105 ºC and weighted to calculate feed intake. Feed consumption was daily recorded.

Table 2-6 I Main ingredients*, energy, protein and % total fatty acids contents of diet for the nutritional challenge of gilthead seabream juveniles obtained from broodstock fed diets 100% FO, 40%FO-60%LO and 20%FO-80%LO (F, LL and HL) during spawning

Main Ingredients	%	Proximate Composition	(% dry matter)
Fish meal SA[1] 68 super prime	5.0	Crude lipids	21.7
Fish meal alternative protein sources[2]	54.5	Crude protein	45.1
Rapeseed meal cake	11.3	Moisture	9.0
Wheat	6.9	Ash	5.4
Fish oil SA[1]	3.0		
Vegetable oil mix[3]	13.0	**Gross Energy (MJ/kg, as is)**	**22.5**
% total fatty acids			
16:1n-7	2.1	20:5n-3	2.5
18:2n-6	20.3	22:1n-11	0.1
18:3n-3	11.8	22:6n-3	1.7

* Please see (Torrecillas *et al.*)diet code 5FM/3FO for the complete list of feed ingredients.
[1]. South American, Superprime (Feed Service, Bremen, Germany).
[2]. Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
[3]. Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

2.2.3.1.4 Experiment four and five, combined effects of nutritional programming with 18C rich fatty acid diet and genetic selection of the broodstock to improve utilization of vegetable meal and oil diets

In experiment four and five, progeny obtained from broodstock fed with different diets (Table 2-3) were nutritionally challenged using very low FM 5% and FO 3% (Table 2-6). This diet is designed to test the possible effects of nutritional programming of the offspring and feed utilization. The formulations of the challenge diets were shown in Table 2-6. Juveniles from each broodstock were daily four time a day (08:00, 11:00, 14:00 and 17:00) fed until apparent satiation with one of the two different diets in triplicates for 30 days. Feed consumption was daily recorded.

2.3 Samplings

2.3.1 Growth and survival

2.3.1.1 Egg production and spawning quality

Spawning quality was determined before and after feeding the experimental diets for each experiment. At the beginning of the trials mean body weight and total length for females and males was taken. Fishes were randomly assigned to experimental tanks using 1 female and 2 male per tank, except for the selection study where the female to male ratio was 1:1. After placing the brood fish in the experimental tanks, fish were fed with commercial diets, and spawning quality monitored daily. Once similar spawning quality parameters were observed between the tanks ($P>0.05$), tanks were randomly assigned to one of the experimental groups.

The spawning quality parameters were determined using eggs those were collected daily from each tank at around 08:00 h and concentrated in 5 L beakers. Immediately, eggs were transferred to the laboratory and aeration supplied to each to ensure aeration of the eggs through the water column. 5 mL sample was taken by using graduated glass pipet and transferred to a Bogorov chamber. Eggs were counted and observed under a binocular microscope (Leica Microsystems, Wetzlar, Germany) in five replicates to calculate: the total number of eggs, the percentage of fertilised eggs and

determine the morphological characteristics. Egg viability rate, determined as the percentage of morphologically normal eggs at the morula stage and described as transparent, perfectly spherical with clear, symmetrical early cleavages (Fernández-Palacios *et al.*, 2011). After that, some egg randomly was taken and transferred to 96 well ELISA microplates using a micropipette (0.7 mL of seawater and one egg per well). Plates were observed under a binocular microscope to ensure there was a single fertilized egg at each well. These eggs were kept temperature-controlled refrigerator to keep the temperature at constant at 23 °C. By observing the egg from these ELISA plates after 24 and 72 h under a binocular microscope, hatching rate and survival after three dah were calculated as percentages. With these percentage values, the total number of fertilized, viable and hatched eggs and larvae survived after 3 dah calculated per female/kg basis.

2.3.1.2 Larval stages

Growth was determined by measuring total length and dry body weight of the larvae from each group. Total length of 15 to 30 (depending on different experiments) anesthetized larvae from each tank was measured in a Profile Projector (V-12A Nikon, Nikon Co., Tokyo, Japan) at the beginning and 10 days after the experimental feeding. Whole body weight was determined by 3 replicates of 10 starved larvae washed with distilled water and dried in a glass slide at an oven at 110 °C, for approximately 24 h followed by 1 h periods until constant weight was reached.

2.3.1.3 Juvenile stages and nutritional challenges

After fishes were reached the desired age, they were from all experimental groups were weighted individually to a global knowledge of the growth of the fish coming from different broodstock groups. From this weight data, similar weight class from each experimental group were selected. and placed to the experimental tanks.

For this thesis 4 nutritional challenge experiments were done. Fishes were produced in three different years and from different broodstock groups.

2.3.1.3.1 Experiment one, effects of parental diets with decreasing levels of n-3 LC-PUFA juvenile stage when fed the same diets as their parents

To determine the effect of the nutritional stimulus through the broodstock diet on the ability of the progeny to utilise low FO and FM diets, juveniles obtained from broodstock fed the different FO/LO (section 2.2.1.2.1) ratios were nutritionally challenged with diets 100FO, 80LO and 100LO (Table 2-4). Juveniles with a mean wet weight of 44.13±0.49, 47.46±0.18, 47.34±0.25 and 38.36±0.96 g from the progeny of broodstock fed with 100FO, 60LO, 80LO and 100LO, respectively, were randomly distributed into 36 tanks (30 fish/treatment). All tanks (200 L fibreglass cylinder tanks with a conical bottom and painted a light grey colour) were supplied with filtered seawater (37 ppm salinity) at a rate of 100 L/h to assure good water quality during the entire trial. Water entered from the tank surface and drained from the bottom to maintain high water quality, which was tested daily, and no deterioration was observed. Water was continuously aerated (125 mL/min) attaining 6.1±0.3 ppm dissolved O_2. Average water temperature and pH along the trial were 21.6±0.8 °C and 7.82, respectively. Photoperiod was kept at 12h light: 12h dark by fluorescent daylights and the light intensity was kept at 1700 lx (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). Juveniles from each broodstock were daily fed until apparent satiation with one of the three different diets in triplicates for 60 days. Growth was determined by measuring wet body weight after 24h starvation. Before measurements, all fish were anaesthetised with 10 ppm clove oil: methanol (50:50) in seawater. Whole body weight of all fishes from each tank was determined at the beginning and the end of the feeding.

2.3.1.3.2 Experiment two, testing the possible nutritional programming effect through the broodstock nutrition in 3-months-old juveniles those are obtained from parents with fed with increasing levels of FO substitution by LO in diets

To determine the effect of the nutritional stimulus through the broodstock diet on ability of the progenies to utilize low FO and FM diets, juveniles obtained from broodstock fed the different FO/LO ratios were challenged with two diets either high or low in FM and FO (Table 2-5). Juveniles with a mean wet weight of 3.97±0.60 g were

randomly distributed into 24 tanks (80 individuals/tank). All tanks (200 L fiberglass cylinder tanks with conical bottom and painted a light grey color) were supplied with filtered seawater (37 ppm salinity) at a rate of 100 L/h to assure good water quality during the entire trial. Water entered from the tank surface and drained from the bottom to maintain high water quality, which were tested daily, and no deterioration was observed. Water was continuously aerated (125 mL/min) attaining 6.1±0.3 ppm dissolved O_2. Average water temperature and pH along the trial were 24.6±0.6 °C and 7.89, respectively. Photoperiod was kept at 12h light: 12h dark by fluorescent daylights and the light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). Diets were formulated and produced by Biomar company, Denmark and they were isoenergetic, one being high in FO (15.30%) and FM (58%) (FOFM diet), and the other extremely low in FO (3%) and FM (5%) (VOVM diet)(Table 2-5). Thus, the FOFM diet had higher levels of ARA, EPA and DHA, whereas the VOVM diet was higher in oleic acid (18:1n-9), LA (18:2n-6), and ALA (18:3n-3) acids (Table 2-5). Juveniles from each broodstock were daily fed until apparent satiation with one of the two different diets in triplicates for 30 days.

2.3.1.3.3 Experiment three, testing the persistence of nutritional programming on growth, lipid metabolism and utilisation in later developmental stages the offspring are 16-months-old

All fish were obtained from spontaneous spawns of gilthead sea bream broodstock fed 3 diets with 3 levels of FO substitution with LO: 100% FO (F), 40% FO-60% LO (LL), 20%FO-80% LO (HL) (Table 2-4). Offspring from all the groups were fed the same commercial diet during larval rearing, weaning and on growing period until they reached 4 months-old (120 days). At this stage, triplicate groups of juveniles were nutritionally challenged for 1 month with either: a high FM/FO diet (f) or a high VM/LO diet (v) named as “reminder diet” in the present study (Table 2-5). Details of the broodstock feeding (Section 2.2.1.2.2), juvenile nutritional challenge (reminder) (Section 2.2.3.1.2) at 4 months were detailed sections above. After this first nutritional challenge

(reminder), fish were maintained separately in 1000 L tanks and fed the same commercial diet until they were 16-month-old to be used in the present study.

In this experiment, 16-month-old offspring seabreams of homogeneous weight were selected and distributed into 18, 500 L light grey-fiberglass cylinder tanks (2.8 kg/m^3). Each tank contained 30 fish with an initial body weight of 243.2 ± 12.7 g (mean±SD). Tanks were supplied with filtered seawater (37 ppm salinity), which entered from the tank surface and drained from the bottom at a rate of 250 L/h to maintain high water quality, which was tested daily, and no deterioration was observed. O_2 level, water temperature and pH were monitored real time by Miranda aquaculture water quality monitoring system (Innovaqua, Sevilla, Spain). Water was continuously aerated (125 mL/min), attaining an average of 6.8±0.8 ppm dissolved O_2 during the experimental period. Average water temperature and pH along the trial were 24.6±0.6 °C and 7.89, respectively. Natural photoperiod was kept during the whole experimental period (10 h light).

2.3.1.3.4 Experiment four and five, combined effects of nutritional programming with 18C rich fatty acid diet and genetic selection of the broodstock to improve utilization of vegetable meal and oil diets

All the fishes obtained from different broodstock groups (Section 2.2.1.2.2) kept in different tanks but similar conditions in triplicates. Fishes were fed with the same commercial diets through the grow-out period until they reach 6-months-of-age. Then fishes were assigned to experimental tanks in triplicates for each broodstock group. Thus, twelve 500 L capacity tanks were used for the juvenile feeding challenge experiment. All fish were anesthetized with 10 ppm clove oil: methanol (50:50) in sea water prior to measurements for the initial and final samplings. Fish were fasted for 24 hours then individually weighted, total lengths were measured, and fish were assigned to each experimental tank. Fish were fed with experimental diet (Table 2-5) twice a day at 08:00 and 14:00 h for 60 days, except for Sundays. Feed consumption was daily recorded. Growth was determined by measuring wet body weight after 24 h starvation.

Prior to measurements all fish were anesthetized with 10 ppm clove oil: methanol (50:50) in sea water.

2.3.1.4 Growth and feed utilisation parameters estimations

Growth performance and feed utilization were calculated with the following formulas:

Weight gain (%) = (Final biomass – initial biomass) / (initial biomass) × 100.

Specific growth rate (SGR %/day) = [Ln (final weight. g) – Ln (initial weight. g)] / (number of days) × 100.

Feed conversion ratio (FCR) = (total weight of consumed feed. g) / (weight gain. g).

2.4 Analysis

2.4.1 Biochemistry

2.4.1.1 Proximate analysis

2.4.1.1.1 Protein

Proteins were estimated from analysis of the total nitrogen present in the sample, using the Kjeldhal method (AOAC, 1995). Briefly, after the digestion of the sample (between 200 - 500 mg, depending on sample size from different tissues) with concentrated sulphuric acid at a temperature of 400ºC. Then total nitrogen content was determined and converted to total crude protein value by multiplying by the empirical factor 6.25.

2.4.1.1.2 Lipids

Lipids were extracted following the method of (Folch *et al.*, 1957). The method starts taking a sample amount between 50-200 mg and homogenizing it in an Ultra-Turrax (IKA-Werke, T25 BASIC, Staufen Germany,) during 5 min in a solution of 5 mL of chloroform: methanol (2:1) with 0.01% of butylated hydroxytoluene (BHT). The resulting solution was filtered by gravimetric pressure through glass wool and 0.88% KCl added to increase the water phase polarity. After decantation and centrifugation at 2000 rpm during 5 min the watery and organic phases were separated. Once the watery phase

was eliminated, the solvent was dried under nitrogen atmosphere and subsequently total lipids weighed.

Neutral and polar fractions were separated by adsorption chromatography on silica cartridges Sep-pak, (Waters, Milford, MA) as described by (Juaneda and Rocquelin, 1985).

2.4.1.1.3 Fatty acid methyl esters preparation and quantification

Fatty acid methyl esters (FAME) were obtained by acid transmethylation of total lipid with 1% sulphuric acid in methanol following the method of (Christie, 1982). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50 ºC. Afterwards, FAMES were extracted with hexane:diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH_2 Sep-pack cartridges (Waters S.A., Massachussets, USA) as described by (Christie, 1982). FAMES were separated by Gas-Liquid Chromatography (GLC) (Agilent 7820A, CA, USA) in a Supercolvax-10-fused silica capillary column (length:30 mm, internal diameter: 0.32 mm, Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC min^{-1} and then held at 215 ºC for 10 min, following the conditions described in (Izquierdo *et al.*, 1990). Fatty acid methyl esters were quantified by Flame ionization detector (FIED) and identified by comparison with external standards and well-characterized FO (EPA 28, Nippai, Ltd. Tokyo, Japan).

2.4.1.1.4 Moisture and ash

Moisture content was determined by thermal oven drying to constant weight at 110 ºC, with a first 24 h drying period, followed by 1 h periods until weight was nor reduced any further or in case of higher weight observed from the weight obtained previously. Sample weight (between 200 - 500 mg, depending on sample size from different tissues) was recorded before drying and after each drying period, following the cooling in a desiccator. Moisture was expressed as a percentage of the weight according to Official Methods of Analysis (AOAC, 1995).

Ash content was determined by drying the samples (between 200 - 500 mg, depending on sample size from different tissues) in a mufla oven at a temperature of 450ºC until a constant weight was attained (AOAC, 1995).

2.4.2 Molecular studies

Molecular studies in this thesis are focused on mainly to identify expression differences in selected genes of PUFA metabolism and stress resistance. Three different approaches were used to examine the effects of broodstock diets on the progeny. These were real-time polymerase chain reaction (PCR) analysis for experiment 1,2,3 to identify the expressions as fold-change to a control group, digital droplet PCR (ddPCR) for experiment 4 and 5 to measure the absolute gene expression and micro-array for a global view of the lipid metabolism related genes of gilthead sea bream. In the last studies, experiment 4 and 5, with the genome sequence of gilthead sea bream became available which has given more opportunities to analyse different epigenetic mechanisms such as DNA promoter methylation of the genes investigated. Primers used in these studies were presented in Table 2-7.

2.4.2.1 RNA extraction

Tissue samples were taken and preserved directly in RNA Later (Sigma-Aldrich) overnight at 4°C. Then RNA Later was removed, and samples kept at -80°C until RNA extraction and analyses. Digestion of tissues for RNA extractions was done using TRI Reagent (Sigma-Aldrich, Missouri, USA). For the digestion, approximately 100 mg of tissue were weighted, 1 mL of TRI Reagent and four pieces of 1 mm diameter zirconium glass beads, were added to 2 mL volume of Eppendorf tubes. Samples were homogenised using TissueLyzer-II (Qiagen, Hilden, Germany) for 60 seconds with a frequency of 30/s until tissue thoroughly dissolved. 250 µL chloroform was added to homogenised samples and then centrifuged at 12000 G for 15 min at 4 °C for phase separation. The transparent upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane. After that RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen) with the protocol supplied by the manufacturer.

2.4.2.2 Real-time PCR analysis for the fold change gene expression

Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using actin beta (*actb*) as the house-keeping gene in a final volume of 15 µL per reaction well, and 100 ng of total RNA reverse transcribed to complementary DNA (cDNA). Samples, housekeeping gene and were analysed in duplicates. Primer efficiency was tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:100 and 1:1000).

Table 2-7 I Primers and GeneBank accession numbers and reference articles for sequences of target and housekeeping genes

Gene*	Primer Sequence 5'-3' (F) and 5'-3' (R)	GenBank Access No.	Reference
lpl	CGT TGC CAA GTT TGT GAC CTG AGG GTG TTC TGG TTG TCT GC	AY495672	(Benedito-Palos *et al.*, 2014)
ppara	TCT CTT CAG CCC ACC ATC CC ATC CCA GCG TGT CGT CTC C	AY590299	(Benedito-Palos *et al.*, 2014)
elovl6	GTG CTG CTC TAC TCC TGG TA ACG GCA TGG ACC AAG TAG T	JX975702	(Benedito-Palos *et al.*, 2014)
fads2	CGA GAG CCA CAG CAG CAG GGA CGG CCT GCG CCT GAG CAG TT	AY055749	(Izquierdo *et al.*, 2015)
cox2 (ptgs2)	GAG TAC TGG AAG CCG AGC AC GAT ATC ACT GCC GCC TGA GT	AM296029	(Sepulcre *et al.*, 2007)
cpt1	CCA CCA GCC AGA CTC CAC AG CAC CAC CAG CAC CCA CAT ATT TAG	DQ866821	(Boukouvala *et al.*, 2010)
pla2	AGA CCA GCA AGC TCA CAC CAC A TGC TGG CTC ACT GTC ACA CT	CX734981.1	
tnfa	CTC ACA CCT CTC AGC CAC AG TTC CGT CTC CAG TTT GTC G	AJ413189.2	(García-Castillo *et al.*, 2002)
ilb1	AGC GAC ATG GCA CGA TTT C GCA CTC TCC TGG CAC ATA TCC	AJ277166.2	(Pelegrín *et al.*, 2001)
mhc1	CTC CCC AAC CAC GAT GGA A CGT CGT GTT AAG TTT CTG GTC	DQ211540.1	(Cuesta *et al.*, 2006)
mhc2	GAG TTC CTC CCC AAC CAC GAT G GCC GTC GTG TTA AGT TTC TCG TCA	DQ211541.1	(Cuesta *et al.*, 2006)
gr	ACA CCG AAA GCA CTG AGG AGG GGG CTG GAT GGA AGA ACG ACA	DQ486890.1	(Acerete *et al.*, 2007)
hsp70	TTG ACC ATT GAG GAT GGC ATC TCC TTC TTG TAC TTG CGC TTG	EU805481.1	(Avella *et al.*, 2010)
hsp90	GTC ATC CTG CTG TTC GAG ACC CTC CTC TAC GGG AAC GTC GTC	DQ012949.1	
actb	TCT GTC TGG ATC GGA GGC TC AAG CAT TTG CGG TGG ACG	KY388508.1	(Santos *et al.*, 1997)
rlp27	ACA ACT CAC TGC CCC ACC AT CTT GCC TTT GCC CAG AAC TT	AY188520.1	(Laizé *et al.*, 2005)

*__Complete gene names__; *lpl*: Lipoprotein lipase, *ppara*: Peroxisome proliferator-activated receptor alpha, *elovl6*: Elongation of very long chain fatty acids protein 6, *fads2*: Fatty acid desaturase 2, *cox2*: Cyclooxygenase-2, *cpt1*: Carnitina palmitoiltransferasa I, *gr*: Glucocorticoid receptor, *hsp70*: Heat shock protein 70, *hsp90*: Heat shock protein 90, *ilb1*: Interleukin 1, *mhc1*: Major histocompatibility complex class I, *mhc2*: Major histocompatibility complex class II, *pla2*: Thermostable phospholipase A2, *tnfa*: Tumor necrosis factor alpha, *actb*: cytoplasmic actin 1, *rpl27*: ribosomal protein L27

Each gene with housekeeping, primer efficiency and blank samples analysed in one 96 well PCR plate (Bio-rad, Multiplate, CA, USA). Melt curve was performed and amplification of single product was confirmed after each run.

Fold expression of each gene was determined by delta–delta C_T method ($2^{-\Delta\Delta CT}$) introduced by (Livak and Schmittgen, 2001). Efficiency correction during analysis were not performed due only small chances observed in PCR efficiencies which further discussed by (Schmittgen and Livak, 2008). Control sample was obtained from individuals those are obtained from 100% FO fed broodstock and fed by commercial diets through their life and fold expression were calculated accordingly.

2.4.2.3 Digital droplet PCR analysis for the absolute gene expression

Tissue RNA extraction was made using the protocol explained above for the real time PCR. A modified version of the RNA extraction method was used for the blood. The whole blood was taken from the caudal vein of the brood fish for the selection of the individuals. Prior to measurements all fish were anesthetized with 10 ppm clove oil/methanol (50:50) in sea water. 2.5 mL blood was taken with 2.5 mL sterile syringes (Terumo Europe NV, Leuven, Belgium) and transferred to 2.5 mL K 3 EDTA tubes (L.P. Italiana, Milan, Italy), then 500 µl transferred to 2 mL Eppendorf tubes. Whole blood samples were kept on ice during the sampling and were immediately centrifuged at 10000 rpm, 4 °C for 20 minutes. Plasma were separated, and erythrocytes were snap frozen with liquid nitrogen and kept at -80 °C until RNA extraction. Samples were kept in the room temperature until samples were thawed completely. For each 200 µL of blood cells 1 mL of TRI Reagent (Sigma-Aldrich, Misuri, USA) were added into 2 mL Eppendorf tubes. To each tube, four pieces of 1 mm diameter zirconium glass beads added and homogenized using TissueLyzer-II (Qiagen, Hilden, Germany) for 60 seconds with a frequency of 30/s. 250 µL chloroform was added to homogenized samples and then centrifuged at 12000 G for 15 min at 4 °C to phase separation. The transparent upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column where total RNA bonded to a membrane. After that RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen) with the protocol supplied by the manufacturer.

Absolute gene expression analysis was performed with ddPCR using Bio-rad QX200 (Hercules, California, U.S.A.) systems using the same cDNA obtained through the steps explained above. Samples were prepared workflow provided by the manufacturer. In summary, for each gene tested master mixes were prepared including 10 µL EvaGreen super mix (Bio-rad, Hercules, California, U.S.A.), 0.2 µL forward primer, 0.2 µL reverse primer, 7.6 µL MilQ water and 2 µL cDNA (approx. 20 ng cDNA). Then droplets were generated using droplet generator Bio-rad QX200 (Hercules, California, U.S.A.). Generated droplets were transferred to 96 well micro plates for PCR in a thermal cycler (Bio-rad C1000 Touch, Hercules, California, U.S.A.). Following to the PCR amplifications droplets read in droplet reader (Bio-rad QX200, Hercules, California, U.S.A.) to identify the absolute gene expression. Reading lower than 12000 droplets was not used for the gene expression.

2.5 Statistical analysis

The statistical analysis in this thesis were done using the IBM SPSS v23.0.0.2 for Mac (IBM SPSS Inc. Chicago, IL, USA). A significance level of 5% ($P<0.05$) was used for all tests performed. Values presented were generally mean with the standard deviation (SD) if not otherwise stated.

2.5.1 Parametric and non-parametric tests

Prior to analysis of the data, equality of variances was tested by Levene's test and distribution of data by Shapiro-Wilk tests and once met the assumptions, were analysed using a two-way analysis of variance (ANOVA) and/or one-way ANOVA. If the difference were found to be significant ($P<0.05$), a Tukey or Scheffé's method multiple comparison post-hoc test was applied using IBM SPSS v23.0.0.2 for Mac (IBM SPSS Inc. Chicago, IL, USA). A general analysis work-flow was as follows;

Two-way ANOVA analysis were carried depending on the fixed factors used in the experiment (such as broodstock diet – juveniles' diet for experiment 1,2 and 3, broodstock diet and selection for the experiment 4 and 5). If significant differences were observed, data were split into groups based on each fixed factor (broodstock diet, juvenile diet, selection…) and compared with one-way ANOVA. Tukey or Scheffé's post-

hoc multiple comparisons for broodstock and reminder diet, separately, assessed differences between groups.

Prior to analysis of the data, equality of variances was tested by Levene's test and distribution of data by Shapiro-Wilk tests. For the parameters that not showed normal distribution and equality of variances, were analysed by two-way ANOVA using fixed factors. This data then was analysed by Welch's ANOVA then compared with Games-Howell test for identification of differences between groups.

2.5.2 Correlation analysis and figures

Pearson correlation test was performed using R (R Foundation for Statistical Computing, Vienna, Austria). Gene expression figures were created using lattice package (version 0.20-33) (Sarkar, 2008) downloaded from the comprehensive R Archive Network (CRAN) library.

Chapter 3

LC-PUFA profiles in parental diets induce long-term effects on growth, fatty acid profiles, expression of fatty acid desaturase 2 and selected immune system-related genes in the offspring of gilthead seabream

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Abstract:

The present study investigated the effects of nutritional programming through parental feeding on offspring performance and expression of selected genes related to stress resistance in a marine teleost. Gilthead seabream broodstock were fed diets containing various fish oil (FO) / vegetable oil (VO) ratios to determine their effects on offspring performance along embryogenesis, larval development and juvenile on-growing periods. Increased substitution of dietary FO by linseed oil (LO) up to 80% LO significantly reduced the total number of eggs produced by kg/female/per spawn. Moreover, at 30 days after hatching (dah), parental feeding with increasing LO up to 80% led to up-regulation of fads2 that was correlated with the increase in conversion rates of related PUFA. Besides, cox2 and tnf-a gene expression were also up-regulated by the increase in LO in broodstock diets up to 60% or 80%, respectively. When four-months old offspring were challenged with diets having different levels of FO, the lowest growth was found in juveniles from broodstock fed 100% FO. Increase in LO levels in the broodstock diet up to 60LO raised LC-PUFA levels in the juveniles, regardless of the juvenile's diet. The results showed that it is possible to nutritionally program gilthead seabream offspring through modification of the fatty acid profiles of parental diets to improve the growth performance of juveniles fed low FO diets, inducing long-term changes in PUFA metabolism with up-regulation of fads2 expression. This study provided the first pieces of evidence of the up-regulation of immune system-related genes in the offspring of parents fed increased FO replacement by LO.

Keywords: *fads2* ; n-3 LC-PUFA biosynthesis; effects of parental nutrition; stress response

3.1 Introduction

The beneficial roles of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA, $>C_{20}$) in particular of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) for retinal and brain development during infancy, prevention of autoimmune disorders and some types of cancers and cardiovascular diseases are well recognised (Connor, 2000; Simopoulos, 2008). EPA and DHA are primarily provided by seafood, whereas these fatty acids are mostly absent from terrestrial foods (Mozaffarian and Wu, 2012). Thus, health organisations worldwide recommend the consumption of fish or food supplements rich in EPA and DHA such as fish oil (FO) (Health and Services, 2015). However, FOs are mostly obtained from capture-derived oily fish, a limited resource whose production has remained steady during the last decade (FAO, 2016). Besides, fish have a limited ability to synthetize EPA and DHA from the precursor α-linolenic acid (ALA; 18:3n-3). Bioconversion of 18 carbon PUFAs to EPA and DHA depends on the elongation and desaturation capacity of different fish species (Sargent *et al.*, 2002; Castro *et al.*, 2016). Generally speaking, freshwater fish possess a higher ability to convert ALA and LA into LC-PUFA (Sargent *et al.*, 1999; Leaver *et al.*, 2008), than marine fish (Tocher, 2010). Moreover, LC-PUFA synthesis capacity also appears to differ among different marine species (Monroig *et al.*, 2012; Monroig *et al.*, 2013; Navarro-Guillén *et al.*, 2014; Xu *et al.*, 2014). For instance, gilthead sea bream (*Sparus aurata*), shows very low desaturation and elongation activity to produce EPA and DHA from ALA (Tocher and Ghioni, 1999). A fatty acyl desaturase 2 gene (*fads2*) has been cloned from gilthead sea bream (Seiliez *et al.*, 2003), encoding Δ6 desaturase (rate-limiting in LC-PUFA synthesis) and being regulated by the diet (Izquierdo *et al.*, 2008). Since some terrestrial vegetable oils (VO) are rich in ALA and constitute a more sustainable feedstuff than FO, recent studies have tried to enhance the ability of gilthead seabream to synthesise EPA and DHA, namely, to up-regulate *fads2* expression and improve dietary lipids utilisation (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017; Turkmen *et al.*, 2017).

In mammals, changes in dietary fat levels and quality during early developmental stages modulate metabolism in later life stages (Ainge *et al.*, 2010; Burdge and Lillycrop, 2010; Lillycrop and Burdge, 2018) through a process that is known as nutritional programming. For instance, feeding rats a high-saturated fat diet during pregnancy and lactation reduces DHA synthesis capacity and decreases mRNA expression of *Fads2* and induced persistent changes in the LC-PUFA profiles of the offspring fed ALA or linoleic acid (18:2n-6; LA) (Kelsall *et al.*, 2012). Moreover, *Fads2* mRNA expression is negatively correlated with CpGs methylation in the promoter of this gene, denoting that epigenetic regulation of *Fads2* may contribute to the long-term regulation of LC-PUFA synthesis in the offspring (Hoile *et al.*, 2013). Additional studies in rodents showed that dietary PUFAs exert epigenetic changes that may lead to differential expression and phenotypes (Lillycrop and Burdge, 2018). Similarly, in gilthead seabream, parental feeding with progressively increased levels of ALA and LA and reduced EPA and DHA significantly up-regulated *fads2* expression in the offspring (Izquierdo *et al.*, 2015). Besides, offspring were better able to utilise dietary fats with low EPA and DHA and high ALA and LA, as it was denoted by their improved growth when fish were fed VO (Izquierdo *et al.*, 2015). Moreover, this improved growth was persistent along fish life almost until reproduction, being related to the improved utilisation of dietary lipids as denoted by the regulation of lipoprotein lipase (*lpl*), carnitine palmitoyltransferase 1 (*cpt1*) and peroxisome proliferator-activated receptor alpha (*ppara*) (Turkmen *et al.*, 2017). Besides, recent studies highlight the epigenetic basis of these changes in the offspring that broodstock diets can alter the DNA methylation status of the *fads2* promoter in the liver, whereas global methylation in this tissue was similar (Turkmen et al., in prep).

However, little is known on the effect of fat quality of parental feeding on offspring health aspects such as immune system functioning or stress resistance. Dietary fatty acids play an important role in human (Calder, 2006; Yaqoob and Calder, 2007; Simopoulos, 2008) and fish health (Montero and Izquierdo, 2010). For instance, dietary fats quantity and quality have a profound effect on immune system functioning (Calder, 1996; Calder, 2002; Yaqoob, 2004; Calder, 2006; Yaqoob and Calder, 2007). Thus, dietary n-6 fatty acids, high in many VO, stimulate the production of pro-inflammatory

eicosanoids and cytokines in terrestrial animals (Calder, 2006) and fish (Secombes *et al.*, 1996; Montero and Izquierdo, 2010), whereas dietary DHA and EPA suppress the production of those pro-inflammatory substances (Calder, 2005). Among other physiological mechanisms, inflammation processes are mediated by the activation of phospholipases A2 (PLA2) that release LC-PUFA from membrane phospholipids and cyclooxygenases (COX) that produce eicosanoids from those fatty acids (Izquierdo and Koven, 2011). Although COXs have a great affinity for arachidonic acid (AA) yielding prostaglandins (PG) from 2-series, EPA is also a good substrate for COX producing 3-series PGs and other eicosanoids that are less potent than AA derivatives and play an important anti-inflammatory role, through mediators termed E-series resolvins (Serhan, 2006). DHA-derived D-resolvins also have anti-inflammatory properties (Marcheselli *et al.*, 2003). Thus, AA-derived eicosanoids enhance the production of inflammatory cytokines such as TNF-alfa and IL-1 (Calder, 2006; Montero *et al.*, 2015) that are the first cytokines up-regulated after injuries or infection, whereas EPA and DHA reduce chronic inflammation by indirectly down-regulating both cytokines (Farooqui *et al.*, 2007). Effects of the LC-PUFAs on the recognition of specific antigens or pathogens have been less studied. For instance, the cells surface proteins, major histocompatibility complex I (MHCI) and II (MHCII) are up-regulated by AA-derived prostaglandins in mammals and fish (Hwang *et al.*, 2000; Torrecillas *et al.*, 2017).

Dietary LC-PUFAs markedly improve stress resistance in fish (Montero and Izquierdo, 2010). Thus, the increase of DHA and EPA in fish larvae improve resistance to handling or acute thermal stress (Liu *et al.*, 2002) and, in juveniles, prevent elevation of cortisol levels during chronic stress (Montero *et al.*, 1998). Also, cortisol release by head kidney cells after stimulation with adrenal corticotrophin hormone (ACTH) is modulated by PUFA through LOX products (Ganga *et al.*, 2006). Moreover, these lipids may bind to glucocorticoid receptors (GR), modulating the action of cortisol and its gene expression (Montero *et al.*, 2015). Besides, LC-PUFA also regulate the expression of mitochondrial and cytoplasmic chaperons such as HSP70 and HSP90, which are necessary for the assembly and maintenance of GR, increasing the binding capacity of this steroid receptor (Benedito-Palos *et al.*, 2016). To our knowledge, there is no

published information on the effect of parental feeding with different types of oils on the expression of these genes in the offspring.

Previous studies showed that broodstock nutrition in gilthead sea bream can induce a nutritional programming effect on the offspring and alter the expression of lipid metabolism-related genes such as *fads2*, *elovl6* and *cpt1* which can improve the utilisation of VO sources during the early (Izquierdo *et al.*, 2015) and later juvenile stage (Turkmen *et al.*, 2017). However, health and stress-related responses to high VO diets in the larval stage, larval growth and utilisation of the similar diets used as a nutritional programming tool was not yet studied. The objective of the present study was to determine the potential persistence effect of parental feeding on offspring performance along life spam and expression of selected genes related to health and stress resistance. For that purpose, gilthead seabream broodstock were fed diets containing various FO/VO ratios to determine their effects on egg and offspring liver fatty acids profiles and performance along embryogenesis, larval development and juvenile on-growing periods. Afterwards, juveniles were submitted to a nutritional challenge feeding them diets containing different FO/VO ratios.

3.2 Materials and Methods

All the mentioned experiments below were conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at Fundación Canaria Parque Científico Tecnológico (FCPCT), University of Las Palmas de Gran Canaria (Canary Islands, Spain).

3.2.1 Parental feeding

A total of thirty-six gilthead seabream brood fish, ranging between 2 and 4 years of age, were randomly selected from another broodstock group of 120 fishes in total. Selected individuals were randomly allocated to twelve 1000 L capacity round fibreglass tanks with a ratio of 2 males and 1 female per tank. At the beginning of the experiment, the average weight and furcal length of the females were 1.35±0.19 kg and 39.7±2.4 cm, while males were 1.02±0.08 kg and 35.8±0.7 cm. Broodstock feeding was conducted in tanks with a flow-through system filled with filtered seawater (37.0±0.5‰ salinity) at a

renewal of 100% per hour and with strong aeration. Water temperature was ambient and ranged between 19.6±0.2 and 21.3±0.3°C. Tanks received non-direct sunlight and the light period ranged between 10 - 12 h between January and May. Since gilthead seabream continues spawning for over 90 days, broodstock fishes were firstly acclimated to the experimental tanks and fed with commercial diets (Skretting, Norway) from January 7 to February 24 and spawning were monitored. Since spawning quality parameters of broodstock kept in different tanks did not differ, tanks were randomly assigned to the different diet groups and fishes were fed with one of the four experimental diets (Table 3-1) from February 25 to May 3. Fish were fed twice a day (08:00 and 15:00 h) with a daily ration of 1% initial biomass of each corresponding experimental tank. Egg samples of all spawns per tank were collected for biochemical analysis during the initial period fed commercial diet and the second period of feeding the broodstock with experimental diets. Offspring from all the groups experimental groups were fed the same type of diets (commercially available) during the whole period from mouth opening until the nutritional challenge test. A schematic view of this nutritional programming history is shown in Figure 3-1.

Broodstock diets were formulated to have different proportions of FO and linseed oil (LO): 100FO, 40FO/60LO, 20FO/80LO and 100LO (Table 3-1). Substitution of FO in broodstock diets by LO raised the 18C fatty acid that are precursors of *fads2*, such as ALA and linoleic acid (LA; 18:2n-6), and reduced the 20 and 22C end products of fatty acid desaturation, such as AA, EPA and DHA (Table 3-2).

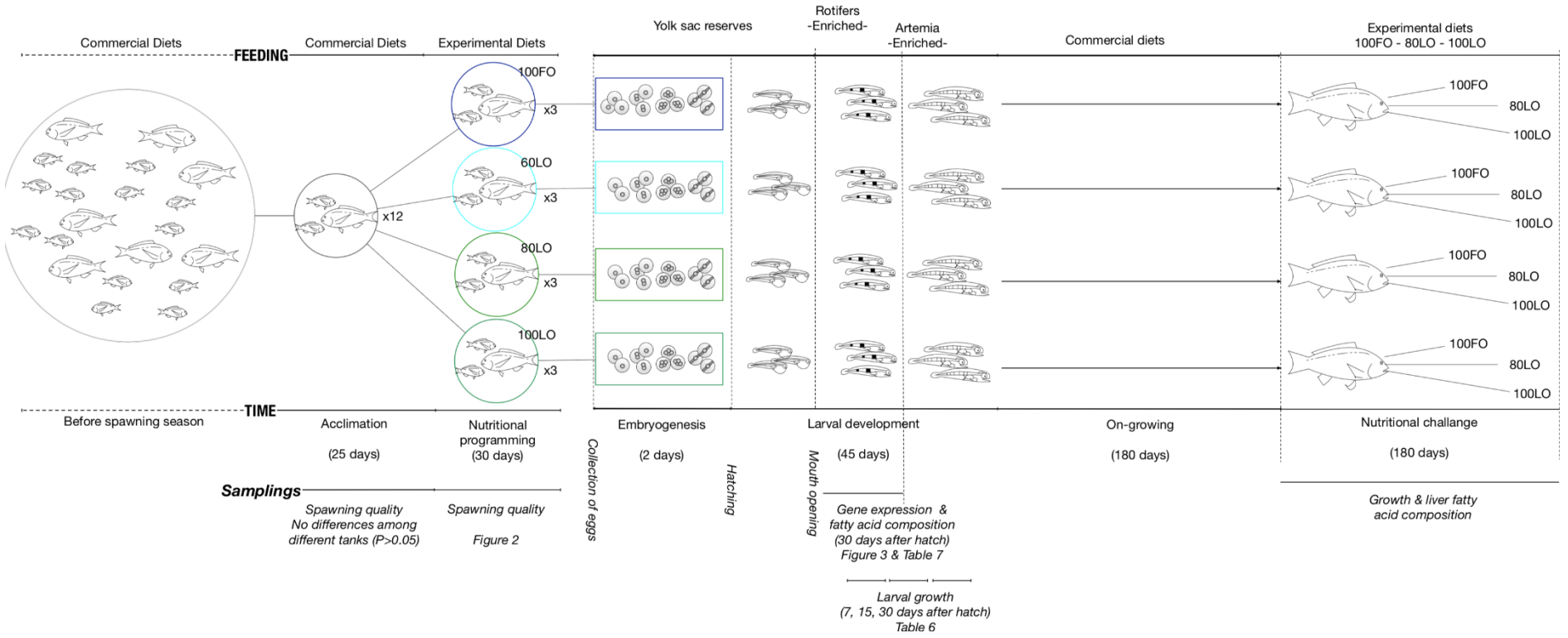

Figure 3-1 I Schematic view of nutritional programming history of gilthead sea bream

Table 3-1 | Main ingredients, proximate composition and energy contents in the practical diets used for the nutritional programming of gilthead seabream broodstock fed different substitution levels of fish oil by vegetable oils

Main ingredients (%)	FO	60LO	80LO	100LO
Marine meals[1]	50.0	50.0	50.0	50.0
Sunflower cake	13.2	13.2	13.2	13.2
Soya cake[2]	10.0	10.0	10.0	10.0
Wheat gluten[3]	9.9	9.9	9.9	9.9
Corn gluten[4]	7.0	7.0	7.0	7.0
Fish oil[5]	*8.0*	*3.2*	*1.6*	-
Linseed oil[6]	-	*4.8*	*6.4*	*8.0*
Vitamin and mineral premix[7]	1.0	1.0	1.0	1.0
Proximate composition (%)				
Moisture	7.73	8.09	7.98	8.08
Crude protein	52.9	53.6	54.5	53.3
Crude lipids	19.5	19.1	19.1	19.5
Ash	8.1	8.4	8.3	8.0
Digestible energy (MJ/kg)	21.2	21.2	21.2	21.2

1 Contains Fish meal NA LT 70, Fish meal SA 68, Feed Service Bremen, Germany.
2 48 Hi Pro Solvent Extra. Svane Shipping, Denmark
3 Hedegaard, Denmark
4 Cargill, Netherlands
5 South American fish oil, LDN Fish Oil, Denmark
6 Ch. Daudruy, France
7 Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany)

3.2.2 Offspring production and quality during embryogenic stages

The amount and quality of the eggs and embryos produced were daily determined before and after feeding the experimental diets as explained above. For that, spontaneously spawned eggs obtained from each experimental broodstock were collected during the whole experimental period. Spawning occurred naturally in separate tanks with 2 males and 1 female as explained above. Eggs were collected from each tank in 5 L beakers every day at 08:00h and transferred to the laboratory with aeration. Five samples of 5 mL were taken by graduated glass pipet and were counted in a Bogorov chamber under a binocular microscope (Leica Microsystems, Wetzlar,

Germany) to calculate: the total number of eggs, the percentage of fertilised eggs and to determine the morphological characteristics.

Table 3-2 l Main fatty acids of practical diets used for the nutritional programming of gilthead seabream broodstock fed different substitution levels of fish oil by vegetable oils (% total fatty acids)

	100 FO	60 LO	80 LO	100LO
FA(% of total)				
14:0	5.90	3.45	2.61	1.97
14:1n-5	0.26	0.15	0.11	0.08
14:1n-7	0.06	0.04	0.03	0.02
15:0	0.47	0.28	0.21	0.15
15:1n-5	0.04	0.03	0.03	0.02
16:0iso	0.11	0.06	0.04	0.03
16:0	17.92	13.48	11.77	10.74
16:1n-7	5.98	3.51	2.73	2.09
16:1n-5	0.23	0.14	0.11	0.08
16:2n-6	0.36	0.20	0.13	0.08
16:2n-4	0.34	0.30	0.30	0.30
17:0	0.29	0.17	0.13	0.10
16:3n-4	0.30	0.18	0.13	0.09
16:3n-3	0.19	0.12	0.09	0.07
16:3n-1	0.07	0.04	0.03	0.04
16:4n-3	0.48	0.30	0.26	0.19
16:4n-1	-	0.01	0.02	0.01
18:0	3.11	3.33	3.31	3.45
18:1n-9	15.31	15.72	15.65	16.18
18:1n-7	3.06	2.27	1.97	1.73
18:1n-5	0.29	0.18	0.14	0.10
18:2n-9	0.06	0.03	0.03	0.03
18:2n-6	6.46	11.23	13.20	14.85
18:2n-4	0.17	0.10	0.07	0.04
18:3n-6	0.15	0.09	0.06	0.04
18:3n-4	0.07	0.06	0.04	0.03
18:3n-3	1.50	21.36	28.79	34.97
18:3n-1	0.01	0.04	0.02	0.03
18:4n-3	2.15	1.15	0.78	0.46
18:4n-1	0.11	0.06	0.04	0.03
20:0	0.11	0.09	0.10	0.09
20:1n-9	0.51	0.30	0.23	0.16
20:1n-7	4.81	3.20	2.51	1.97
20:1n-5	0.32	0.19	0.13	0.09
20:2n-9	0.06	0.03	0.01	0.01
20:2n-6	0.29	0.20	0.11	0.07
20:3n-9	0.04	0.03	0.03	0.02
20:3n-6	0.09	0.05	0.04	0.02
20:4n-6	0.63	0.40	0.32	0.23
20:3n-3	0.15	0.11	0.10	0.05
20:4n-3	0.62	0.32	0.23	0.13
20:5n-3	8.79	5.55	4.56	3.18
22:1n-11	6.32	3.77	2.67	1.80
22:1n-9	0.69	0.45	0.37	0.29
22:4n-6	0.08	0.05	0.04	0.03
22:5n-6	0.07	0.04	0.02	0.01
22:5n-3	1.06	0.58	0.39	0.21
22:6n-3	9.91	6.56	5.32	3.64
Saturated	27.80	20.81	18.12	16.49
n-3	24.85	36.07	40.51	42.91
n-6	8.14	12.25	13.93	15.33
n-9	16.62	16.53	16.28	16.68
EPA/AA	13.95	13.88	14.25	13.83
DHA/AA	15.73	16.40	16.63	15.83
n-3 LC-PUFA	20.52	13.13	10.60	7.22

Egg viability rates were defined as the percentage of morphologically normal eggs at the morula stage and described as transparent, perfectly spherical with clear, symmetrical early cleavages (Fernández-Palacios *et al.*, 2011). After that, fertilised eggs were individually incubated in 96-well ELISA microplates using a micropipette. These eggs were kept at a constant 23 °C temperature. After 24 and 72 hours, rates of hatching and survival after three days were counted under a binocular microscope. With these percentage values, the total number of fertilised, viable and hatched eggs and larvae survived after 3 dah were calculated per female/kg basis.

3.2.3 Offspring performance during larval stages

After one month of feeding the broodstock with the experimental diets, fertilised eggs from individual tanks were stocked into separate mass production tanks (2000 L) at a density of 100 eggs/mL and larvae produced under the same rearing protocol, each parental diet group, was produced in triplicates. In brief, sand filtered, and UV sterilised seawater flow was progressively increased from 10 to 40% per h until 46 dah. Water was continuously aerated (125 mL/min) attaining 6.1 ± 0.4 ppm dissolved O_2; average water temperature and pH along the trial were 20.8 ± 1.3 °C and 7.89 ± 0.6, respectively; a 1500–3500lx 12h single central light (Mod TLD 36 W/54, Philips, France) photoperiod was provided and living phytoplankton (*Nannochloropsis sp.*) (250 ± 100 × 103 cells per mL) was added to the rearing tanks. Regarding feeding, from 3–32 dah, larvae were fed twice a day with rotifers (*Brachionus plicatilis*) at 5–10 rot/mL enriched using emulsions which are commercially available (ORI-GREEN, Skretting, Norway); from 15–17 dah, *Artemia sp.* enriched using the same emulsions as rotifers were added three times a day; and from 20 dah, larvae were fed commercial diets (Gemma Micro, Skretting, France). At 5, 15 and 30 dah, growth was determined by measuring total length and body weight of 30 larvae anaesthetised with clove oil.

3.2.4 Nutritional challenge of juveniles

To challenge the ability of the progeny to utilise low FO diets, four months old juveniles obtained from broodstock groups fed the different FO/LO ratios and reared until days as explained above, were fed either a positive control diet 100FO or the two

most challenging diets 80LO and 100LO (Table 3-1). Diet 60LO was not tested since a marginal reduction in n-3 LC-PUFA (around 2.3% n-3 LC-PUFA in dry diet) would be more than sufficient to fulfil the essential fatty acid requirements of gilthead seabream juveniles (Ibeas *et al.*, 1994). Juveniles with a mean wet weight of 44.1±0.5, 47.5±0.2, 47.3±0.3 and 38.4±1.0 g from the progeny of broodstock fed with 100FO, 60LO, 80LO and 100LO, respectively, were randomly distributed into 36 tanks (30 fish/treatment). All tanks (200 l fibreglass cylinder tanks with a conical bottom and painted a light grey colour) were supplied with filtered seawater (37 ppm salinity) at a rate of 100 L/h to assure good water quality during the entire trial. Water entered from the tank surface and drained from the bottom to maintain high water quality, which was tested daily, and no deterioration was observed. Water was continuously aerated (125 ml/min) attaining 6.1±0.3 ppm dissolved O_2. Average water temperature and pH along the trial were 21.6±0.8°C and 7.82, respectively. Photoperiod was kept at 12h light: 12h dark by fluorescent daylights and the light intensity was kept at 1700 lx (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). Juveniles from each broodstock were daily fed until apparent satiation with one of the three different diets in triplicates for 60 days. Growth was determined by measuring wet body weight after 24h starvation. Before measurements, all fish were anaesthetised with 10 ppm clove oil: methanol (1:1 v/v) in seawater. Whole body weight of all fishes from each tank was determined at the beginning and the end of the feeding trial.

3.2.5 Proximate and fatty acid analysis

Diets, spawned eggs, whole larvae and liver of juveniles were analysed for proximate and fatty acid composition. Moisture, protein (AOAC, 1995) and crude lipid (Folch *et al.*, 1957) contents of the eggs, larvae, juveniles and diets were analysed. Fatty acid methyl esters (FAMES) were obtained by trans-methylation of crude lipids as previously described (Christie, 1982). FAMES were separated using gas chromatography (GC-14A; Shimadzu) following the conditions described previously (Izquierdo *et al.*, 1990) and identified by comparison with previously characterised standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific).

3.2.6 Molecular studies

At 30 dah, around 100 mg of unfed sea bream larvae obtained from broodstock groups fed the different FO/LO ratios were collected from each tank and preserved in 500 µl of RNA Later (Sigma-Aldrich) overnight at 4°C. Then RNA Later was removed, and samples kept at -80°C until RNA extraction and analyses. Tissue digestion for RNA extractions was done using TRI Reagent (Sigma-Aldrich, Missouri, USA). For that purpose, approximately 75 mg of larvae were weighted, 1 mL of TRI Reagent and four pieces of 1mm diameter zirconium glass beads, were added to 2 mL volume of Eppendorf tubes. Samples were homogenised using TissueLyzer-II (Qiagen, Hilden, Germany) for 60 seconds with a frequency of 30/s until tissue thoroughly dissolved. 250 µl chloroform was added to homogenised samples and then centrifuged at 12000 G for 15 min at 4 °C for phase separation. The transparent upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column where total RNA bonded to a membrane. After that RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen) with the protocol supplied by the manufacturer. Before real-time PCR analysis two different potential housekeeping genes, β-actin (*acbt*) and ribosomal protein L27 (*rpl27*), were tested and stability across experimental groups were confirmed. Real-time quantitative PCR was performed in an iQ5 Multi-color Real-Time PCR detection system (Bio-Rad) using *acbt* and *rpl27* as the housekeeping gene in a final volume of 15 µl/reaction well and with 100ng of total RNA reverse-transcribed to complementary DNA (cDNA). Samples, housekeeping genes, cDNA template and reaction blanks were analysed in duplicates (Table 3-3). Primer efficiency was tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:100 and 1:1000). Ninety-six-well PCR plate was used to analyse each gene, primer efficiency and blank samples (Multiplate; Bio-Rad). Melting-curve analysis was performed, and amplification of a single product was confirmed after each run. Fold expression of each gene was determined by delta-delta CT method ($2^{\Delta\Delta CT}$)(Livak and Schmittgen, 2001). PCR efficiencies were similar, and no efficiency correction was required(Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Fold expression was related to that of offspring obtained from FO diet-fed broodstock (100FO).

3.2.7 Statistical analysis

The results were expressed as the mean ± standard deviation (n=3) if not otherwise stated in the tables and figures. The data were compared statistically using the analysis of variance (ANOVA), at a significant level of 5%. For the juvenile nutritional challenge test, due to the differences in the initial weights, this parameter was taken as a covariate in the statistical analysis. All variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the Levene tests, respectively (Sokal and Rohlf, 1969). If significant differences were detected with ANOVA, means were compared by a Tukey test. All data were analysed using IBM SPSS v23.0.0.2 for Mac (IBM SPSS Inc. Chicago, IL, USA).

Table 3-3 I Primers, GeneBank accession numbers and reference articles for sequences of target and housekeeping genes

Gene*	Primer Sequence 5'-3' (F) and 5'-3' (R)	GenBank Access No.	Reference
fads2	CGA GAG CCA CAG CAG CAG GGA CGG CCT GCG CCT GAG CAG TT	AY055749	(Sepulcre *et al.*, 2007)
pla2	AGA CCA GCA AGC TCA CAC CAC A TGC TGG CTC ACT GTC ACA CT	CX734981.1	
cox2	GAG TAC TGG AAG CCG AGC AC GAT ATC ACT GCC GCC TGA GT	AM296029	(Sepulcre *et al.*, 2007)
tnfa	CTC ACA CCT CTC AGC CAC AG TTC CGT CTC CAG TTT GTC G	AJ413189.2	(García-Castillo *et al.*, 2002)
ilb1	AGC GAC ATG GCA CGA TTT C GCA CTC TCC TGG CAC ATA TCC	AJ277166.2	(Pelegrín *et al.*, 2001)
mhc1	CTC CCC AAC CAC GAT GGA A CGT CGT GTT AAG TTT CTG GTC	DQ211540.1	(Cuesta *et al.*, 2006)
mhc2	GAG TTC CTC CCC AAC CAC GAT G GCC GTC GTG TTA AGT TTC TCG TCA	DQ211541.1	(Cuesta *et al.*, 2006)
gr	ACA CCG AAA GCA CTG AGG AGG GGG CTG GAT GGA AGA ACG ACA	DQ486890.1	(Acerete *et al.*, 2007)
hsp70	TTG ACC ATT GAG GAT GGC ATC TCC TTC TTG TAC TTG CGC TTG	EU805481.1	(Avella *et al.*, 2010)
hsp90	GTC ATC CTG CTG TTC GAG ACC CTC CTC TAC GGG AAC GTC GTC	DQ012949.1	
actb	TCT GTC TGG ATC GGA GGC TC AAG CAT TTG CGG TGG ACG	KY388508.1	(Santos *et al.*, 1997)
rlp27	ACA ACT CAC TGC CCC ACC AT CTT GCC TTT GCC CAG AAC TT	AY188520.1	(Laizé *et al.*, 2005)

Complete gene names; *cox2*: Cyclooxygenase-2, *fads2*: Fatty acid desaturase 2, *gr*: Glucocorticoid receptor, *hsp70*: Heat shock protein 70, *hsp90*: Heat shock protein 90, *ilb1*: Interleukin 1, *mhc1*: Major histocompatibility complex class I, *mhc2*: Major histocompatibility complex class II, *pla2*: Thermostable phospholipase A2, *tnfa*: Tumor necrosis factor alpha, *actb*: cytoplasmic actin 1, *rpl27*: ribosomal protein L27a

3.3 Results

3.3.1 Parental feeding and offspring production and performance during embryonic stages

Before the nutritional stimulus through the broodstock diet when all fish were fed with the same commercial diet, no significant (P>0.05) differences were found in the total number of eggs produced by kg female per spawn (59996.10±31209.00) or any spawning quality parameters (96.68±5.03% fertilisation, 62.95±17.57% viable eggs, 95.90±5.93% hatching and 54.35±17.88% three day-old larvae) (mean±SD). However, during the nutritional stimulus, increased substitution of dietary FO by LO up to 80-100% LO significantly reduced the total number of eggs produced by kg female per spawn, whereas 60% substitution did not affect this quality parameter (Figure 3-2).

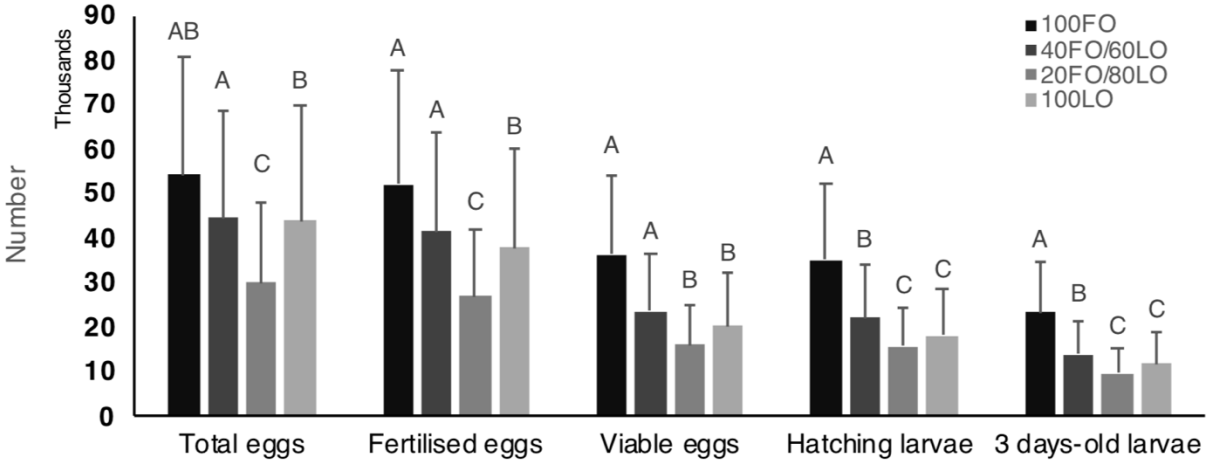

Figure 3-2 | Spawning quality in broodstock fed diets with the progressive substitution of FO by LO during four months of spawning (n = 105). Different letters denote differences (P<0.05). Error bars indicate standard deviations of the means.

The total number of fertilised eggs followed the same tendency as total eggs (Figure 3-2) since fertilisation rates were not affected by the broodstock diet (P>0.05). Besides, the inclusion of LO up to 80-100% markedly reduced the percentage of viable eggs and consequently, the total number of viable eggs and hatched larvae were significantly lower when broodstock was fed with these diets than with the 100FO or 40FO/60LO diets (Figure 3-2). Finally, survival of 3 dah larvae was also markedly reduced when broodstock was fed with 20FO/80LO and 100LO (Figure 3-2).

Before the nutritional stimulus through the broodstock diet, during all groups were fed with the same commercial diet, eggs proximate composition did not significantly differ among the different broodstock (average values of 4 groups (%), protein: 58.83±4.46, lipid: 24.13±1.09, moisture: 88.45±0.21, ash: 17±0.69, $P>0.05$). Besides, there were no significant differences in the fatty acid composition of the eggs obtained from the broodstock during this adaptation period before feeding the experimental diets (LA: 7.67±1.40, ALA: 6.78±1.96, AA: 0.96±0.17, EPA: 7.32±1.81, DHA: 15.00±1.92, % of total fatty acids, average values of all experimental groups, n=4). On the contrary, feeding broodstock the different FO/LO diets, although did not affected eggs proximate composition (% of total, protein: 58.84±4.47, lipid: 24.17±1.07, moisture: 88.52±0.22, ash:17.05±0.67, average values of all experimental groups, n=4) significantly modified fatty acid profiles (Table 3-4). Thus, dietary FO substitution by LO significantly ($P<0.05$) reduced the egg contents in saturated fatty acids, particularly 14:0, 15:0 and16:0, monoenoic, particularly 14:1 and 16:1, as well as n-3 LC-PUFA, including EPA and DHA (Table 3-4). On the contrary, FO substitution by LO increased the egg contents in ALA, raising the n-3 fatty acid levels, and LA and n-6, particularly in fish fed diets 20FO/80LO and 100LO (Table 3-4). From all the fatty acids identified in seabream eggs, the levels of 10 of them were highly correlated with fatty acid profiles of broodstock diet (Table 3-5).

Table 3-4 | Fatty acid composition (% total fatty acids) of eggs obtained from gilthead sea bream broodstock after feeding broodstock diets with the progressive substitution of FO*

	100FO	60LO	80LO	100LO
FA (% of total)				
14:0	3.59±0.39^{a}	3.01±0.20^{ab}	2.74±0.60^{abc}	1.99±0.19^{c}
14:1n-7	0.04±0.01^{a}	0.03±0.01^{ab}	0.03±0.01^{b}	0.02±0.00^{b}
14:1n-5	0.16±0.02^{a}	0.12±0.02^{b}	0.10±0.02^{bc}	0.06±0.02^{c}
15:0	0.35±0.03^{a}	0.31±0.02^{ab}	0.26±0.04^{bc}	0.18±0.02^{d}
15:1n-5	0.04±0.01	0.04±0.01	0.03±0.01	0.03±0.01
16:0ISO	0.13±0.10	0.09±0.08	0.10±0.10	0.08±0.08
16:0	20.31±0.58^{a}	16.57±0.79^{b}	16.43±0.85^{b}	13.89±0.51^{c}
16:1n-7	5.52±0.04^{a}	4.79±0.47^{b}	4.23±0.89^{ab}	2.62±0.34^{c}
16:1n-5	0.17±0.03^{a}	0.13±0.02^{ab}	0.12±0.02^{b}	0.06±0.03^{b}
16:2n-4	0.26±0.03^{a}	0.28±0.06^{a}	0.24±0.06^{ab}	0.14±0.03^{b}
17:0	0.17±0.02^{b}	0.22±0.06^{a}	0.19±0.04^{ab}	0.10±0.03^{b}
16:3n-4	0.33±0.02^{a}	0.23±0.02^{b}	0.20±0.02^{b}	0.15±0.02^{c}
16:3n-3	0.14±0.02^{a}	0.10±0.02^{b}	0.08±0.01^{b}	0.07±0.01^{b}
16:3n-1	0.10±0.01	0.11±0.02	0.10±0.01	0.09±0.01
16:4n-3	0.14±0.03	0.14±0.04	0.14±0.05	0.06±0.02
16:4n-1	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.01
18:0	4.48±0.26^{ab}	4.27±0.65^{b}	4.47±0.34^{ab}	5.61±0.78^{a}
18:1n-9	16.62±0.26	15.36±1.50	16.20±1.41	16.94±0.60
18:1n-7	3.11±0.13^{a}	2.65±0.08^{b}	2.54±0.29^{bc}	2.12±0.12^{c}
18:1n-5	0.20±0.03^{a}	0.13±0.03^{b}	0.12±0.02^{b}	0.11±0.01^{b}
18:2n-9	0.14±0.08	0.15±0.03	0.17±0.06	0.11±0.03
18:2n-6	5.73±0.32^{d}	9.75±0.49^{c}	10.41±0.84^{bc}	13.05±0.08^{a}
18:2n-4	0.18±0.02^{a}	0.18±0.04^{a}	0.16±0.03^{ab}	0.10±0.01^{b}
18:3n-6	0.20±0.07	0.25±0.03	0.28±0.07	0.19±0.04
18:3n-4	0.17±0.03^{a}	0.15±0.03^{a}	0.14±0.03^{ab}	0.08±0.02^{b}
18:3n-3	1.06±0.17^{c}	12.43±1.14^{b}	13.90±3.70^{b}	20.93±1.39^{b}
18:3n-1	0.01±0.01	0.01±0.00	0.01±0.01	0.00±0.01
18:4n-3	0.84±0.19	0.84±0.09	0.76±0.06	0.52±0.19
18:4n-1	0.12±0.00^{a}	0.12±0.02^{a}	0.11±0.02^{b}	0.06±0.01^{b}
20:0	0.10±0.01	0.10±0.01	0.11±0.03	0.11±0.02
20:1n-9	0.22±0.04^{a}	0.11±0.04^{b}	0.10±0.02^{b}	0.10±0.02^{b}
20:1n-7	1.11±0.20^{a}	0.64±0.20^{b}	0.67±0.20^{ab}	0.67±0.07^{ab}
20:1n-5	0.15±0.01^{a}	0.12±0.01^{ab}	0.11±0.02^{bc}	0.08±0.02^{c}
20:2n-9	0.07±0.02^{a}	0.05±0.01^{ab}	0.05±0.02^{ab}	0.04±0.01^{b}
20:2n-6	0.21±0.03^{ab}	0.20±0.01^{b}	0.25±0.03^{ab}	0.34±0.10^{a}
20:3n-9	0.04±0.01^{a}	0.03±0.01^{a}	0.03±0.01^{b}	0.01±0.01^{b}
20:3n-6	0.14±0.02	0.10±0.01	0.11±0.02	0.11±0.03
20:4n-6	0.92±0.03^{a}	0.79±0.14^{ab}	0.69±0.10^{abc}	0.48±0.01^{c}
20:3n-3	0.17±0.07^{b}	0.46±0.04^{b}	0.63±0.30^{b}	1.24±0.36^{a}
20:4n-3	0.86±0.10^{a}	0.61±0.09^{b}	0.56±0.09^{b}	0.49±0.07^{b}
20:5n-3	8.20±0.56^{a}	6.74±0.04^{b}	6.06±0.53^{bc}	4.51±0.29^{d}
22:1n-11	0.46±0.06^{a}	0.20±0.09^{b}	0.17±0.07^{b}	0.15±0.04^{b}
22:1n-9	0.15±0.02^{a}	0.09±0.01^{b}	0.08±0.03^{b}	0.10±0.02^{ab}
22:4n-6	0.10±0.02^{a}	0.07±0.02^{ab}	0.06±0.00^{ab}	0.04±0.01^{b}
22:5n-6	0.25±0.02^{a}	0.25±0.09^{a}	0.13±0.02^{b}	0.10±0.01^{b}
22:5n-3	2.73±0.40^{a}	2.13±0.46^{ab}	2.06±0.22^{ab}	1.48±0.10^{b}
22:6n-3	19.80±1.85^{a}	14.84±1.26^{b}	13.82±1.68^{bc}	10.56±0.43^{c}
Total				
Saturated	29.46±2.34^{a}	22.96±3.04^{b}	19.80±2.37^{b}	17.43±1.33^{b}
n-3	21.83±4.26^{b}	32.26±5.38^{a}	37.47±4.29^{a}	41.14±2.49^{a}
n-6	7.92±0.31^{d}	12.17±0.11^{c}	13.94±0.01^{b}	15.38±0.07^{a}
n-9	17.21±0.83	17.36±1.17	17.12±1.19	17.31±0.89
EPA/AA	8.90±0.77	8.74±1.42	8.82±0.74	8.98±0.04
DHA/AA	21.51±2.11	19.09±1.69	20.08±1.74	20.31±2.03
n-3 LC-PUFA	17.87±3.74^{a}	11.12±2.83^{b}	9.05±2.18^{b}	6.37±1.20^{b}

* Mean±SD, n=4 (one pool of eggs from all the spawns per broodstock tank), different superscripts for each fatty acid would denote significant differences, P<0.05.

Table 3-5 | Relation between dietary and egg fatty acid content for each given fatty acid or groups of fatty acids (n=12).

Fatty acid	R	P
14:0	0.99	0.01
16:0	0.97	0.05
16:1n-7	0.98	0.05
18:1n-7	0.99	0.05
18:2n-6	0.99	0.01
18:3n-3	0.99	0.01
20:4n-6	0.99	0.01
20:5n-3	0.99	0.01
22:5n-3	0.99	0.05
22:6n-3	0.99	0.01
Total Saturated	0.94	0.10
Total Monoenoic	0.96	0.05
Total n-3	0.99	0.10
Total n-6	0.99	0.01
Total n-9	0.99	0.10
Total n-3 LC-PUFA	0.98	0.05

3.3.2 Offspring performance during larval stages

Even though all larvae underwent the same common rearing protocols with rotifers, Artemia and microbound weaning diets, at 30dah, larval growth in terms of total length ($P<0.05$) was lower in offspring from parents fed 100LO (Table 3-6). Regarding molecular studies, parental feeding with 80% FO substitution by LO (80LO group), increased the relative expression of the *fads2* gene in the 30 dah larvae in comparison to 100FO ($P>0.05$, Figure 3-3), whereas further FO replacement with LO (100LO) in the broodstock diets reduced *fads2* expression that showed intermediate values.

Table 3-6 | The growth of larvae obtained from broodstock fed diets with the progressive substitution of FO by LO and fed the same commercial feeding protocol from first feeding*

	Larval age (dah)	Parental diet			
		100FO	60LO	80LO	100LO
Total length (mm)	7	3.67±0.31	3.75±0.21	3.80±0.43	3.77±0.29
	15	4.75±0.46	4.77±0.45	4.80±0.33	4.65±0.44
	30	8.60±1.50[a]	8.72±1.25[a]	8.72±1.62[a]	7.52±1.65[b]
Dry weight (mg)	7	0.03±0.01	0.03±0.01	0.05±0.02	0.04±0.02
	15	0.10±0.02	0.09±0.02	0.10±0.01	0.08±0.02
	30	0.44±0.04	0.41±0.01	0.45±0.01	0.38±0.06

*Letters indicate differences between fish coming from different parental feeding ($P<0.05$). All values are mean ± SD (n=60).

Expression of *pla2* followed a similar trend but did not show significant differences. *cox2* gene expression level was also up-regulated by the increase in LO in parental diets up to 40FO/60LO ($P<0.05$, Figure 3-3) and did not differ with the expression in larvae from parents fed higher LO levels. Besides, *tnf-alpha* expression was highest in 20FO/80LO larvae when compared with the other ones ($P<0.05$, Figure 3-3). There were no significant differences in the expression of *ilb1*. Although we did not find any significant differences among mean values of *mhc1* and *mhc2* ($P>0.05$), expression of mhc*2* was negatively related to the increase in dietary LO levels in broodstock diets up to 20FO/80LO ($R=-0.82$, $P=0.001$), in contrast to what was observed in *fads2* expression levels. Finally, regarding the stress-related genes, no clear differences were found in the expression of *gr* or *hsp90* ($P<0.05$), whereas *hsp70* expression was higher although not significant, in LO substitution groups than in the other groups ($R=0.48$, $P=0.07$) (Figure 3-3).

Analyses of the fatty acid composition of the 30 dah larvae showed that there were no significant differences in the individual fatty acids among different fish groups ($P>0.05$)(Supplemental Table 1, located at the end of the result section). However, conversion rates of fatty acids from 18:3n-3 to 18:4n-3 and from 20:4n-3 to 20:5n-3 showed a strong correlation with *fads2* gene expression (0.93 and 0.92, respectively) (Table 3-7).

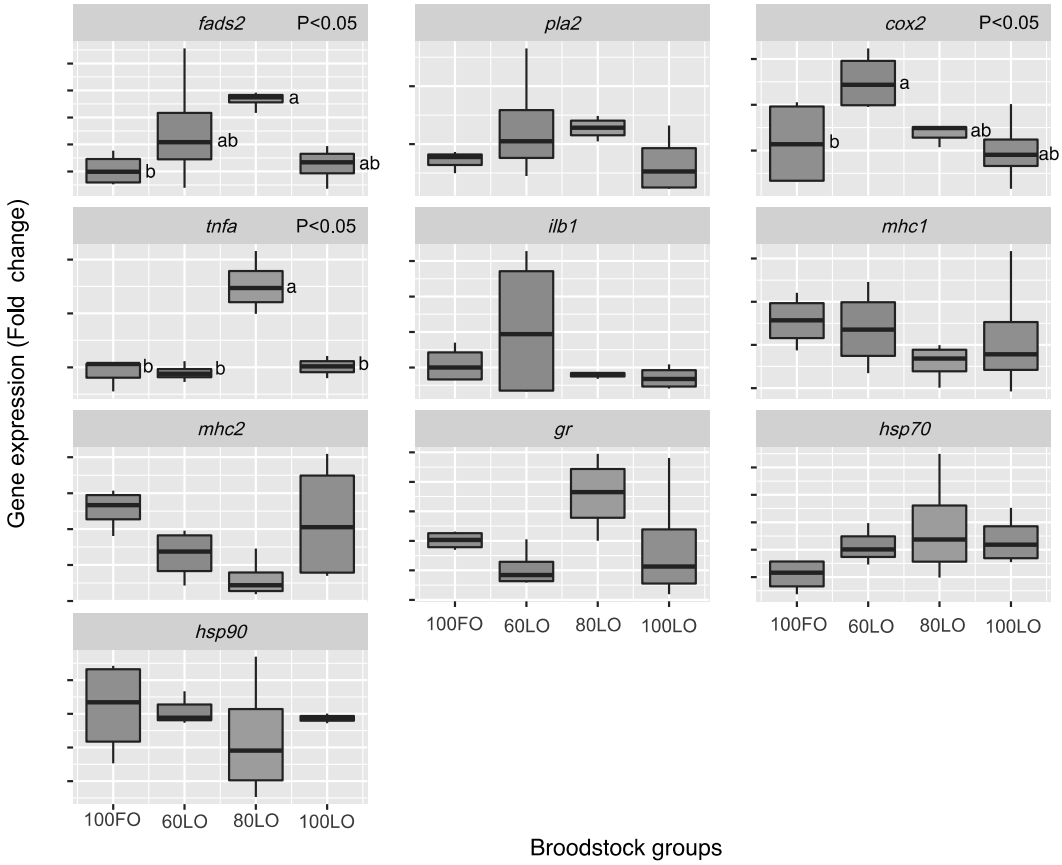

Figure 3-3 I Box-and-whisker plots of relative fold expression of ten different genes after 30 days post-hatch gilthead seabream larvae

Complete gene names; *cox2*: Cyclooxygenase-2, *fads2*: Fatty acid desaturase 2, *gr*: Glucocorticoid receptor, *hsp70*: Heat shock protein 70, *hsp90*: Heat shock protein 90, *ilb1*: Interleukin 1, *mhc1*: Major histocompatibility complex class I, *mhc2*: Major histocompatibility complex class II, *pla2*: Thermostable phospholipase A2, *tnfa*: Tumor necrosis factor alpha
Indications are as follows: Dashed lines = maximum and minimum fold expression, boxes = upper and lower quartiles, and dots = median. n=4 for all the groups and genes. Letters indicate the difference between each group; no indications mean no significance difference (P-value > 0.05).

Besides, correlations with *fads2* expression were lower for 18:2n-6 to 18:3n-6 and 20:3n-6 to 20:4n-6 conversion (Table 3-7). Moreover, end products of LC-PUFA synthesis such as 22:5n-6 (DPA) and DHA were highly correlated with *fads2* expression in 30dah larvae (Table 3-7).

3.3.3 The nutritional challenge of juveniles

Before the beginning of the nutritional challenge, initial weights of juveniles coming from 100LO fed broodstock were smaller than the rest (P<0.05), followed by those coming from broodstock fed 100FO diet (Table 3-8). Therefore, there was a

significant effect of the broodstock diet (family effect) on the initial weight denoted by the two-way ANOVA analysis (P<0.001) (Table 3-8). During the nutritional challenge, the lowest growth was obtained in fish coming from broodstock fed FO diet, whereas FO replacement by LO in broodstock diet enhanced the growth of juveniles (Table 3-8). As a consequence, after 60 days of feeding the nutritional challenge diets, there was a significant effect of the broodstock diet (family effect) on final body weight, weight gain and specific growth rate (SGR), as denoted by the two-way ANOVA analysis (P<0.001) (Table 3-8).

Table 3-7 | Principal substrates and products of delta-6-desaturase enzyme and correlation with *fads2* gene expression at 30 dah larvae obtained from broodstock fed with different diets

			Groups				Correlation with *fads2* expression
Conversion Rate (%)*	Substrate	Product	**100FO**	**60LO**	**80LO†**	**100LO**	
	18:3n-3	18:4n-3	11.13	11.74	-	11.41	**0.93**
	18:2n-6	18:3n-6	3.51	3.60	-	3.14	**0.56**
	20:4n-3	20:5n-3	82.96	83.65	-	83.30	**0.92**
	20:3n-6	20:4n-6	88.90	91.11	-	91.31	**0.53**
Fatty acids	% of total fatty acids						
		22:5n-6	3.47	4.23	-	3.00	**0.89**
		22:6n-3	12.65	14.96	-	11.59	**0.91**

† No data available.
Conversion rates were calculated as 100[product area/(product area + substrate area)]

Broodstock diets and juvenile challenge diets did not significantly (P>0.05) affect the lipid content (Table 3-9). Fatty acid profiles of liver from juveniles after the nutritional challenge were significantly affected by both the juvenile's diet and the broodstock diet (Table 3-9). In particular, increase in LO in the juveniles diet significantly (P<0.001) raised 18:2n-6, 18:3n-6 and 18:3n-3, reflecting the diet contents, whereas the LO increase in the parental diet lead to a general reduction (P<0.001) in those fatty acids that are precursors of LC-PUFA (Table 3-9). Regarding LC-PUFA, there was a general trend towards reduction with the increase of LO in the juveniles diet, EPA, AA or DHA (P<0.001)(Table 3-9), whereas increase in LO levels in the broodstock diet up to 60LO tends to increase AA and DHA (P<0.01) and EPA (P<0.001). However, 20:3n-6 was found to be similar across juvenile dietary treatments (P>0.05)(Table 3-9).

Table 3-8 I Growth performance parameters of juveniles if fed with 3 different diets at 4 months of age for 60 days

Juvenile diet (D)	100FO				80LO				100LO				Two-way ANOVA*		
Broodstock diet (F)	100FO	60LO	80LO	100LO	100FO	60LO	80LO	100LO	100FO	60LO	80LO	100LO	Diet	Family	D*F
Initial Body Weight (g)	44.51±0.13	47.60±1.04	47.09±0.10	37.72±0.58	43.57±1.04	47.52±0.86	47.59±1.85	39.46±0.31	44.33±0.72	47.25±1.76	47.33±0.46	37.88±1.07	-	*	-
Final Body Weight (g)	66.26±3.11	72.74±0.56	75.85±0.88	70.65±2.77	63.32±1.07	70.12±1.72	75.53±2.23	74.65±6.70	66.98±3.30	70.97±2.31	72.09±4.82	67.65±9.53	-	*	-
Weight gain (g)	21.75±3.16	25.14±0.47	28.76±0.53	32.93±2.45	19.75±1.92	22.60±0.14	27.93±1.53	35.18±6.40	22.65±3.29	23.72±1.95	24.76±4.54	29.76±9.61	-	*	-
SGR† (%/day)	0.66±0.08	0.71±0.01	0.79±0.02	1.05±0.05	0.62±0.06	0.65±0.02	0.77±0.03	1.06±0.14	0.69±0.08	0.68±0.04	0.70±0.11	0.96±0.25	-	*	-

***Two-way ANOVA**: Broodstock origin defined as family, diets indicate different diets fed during the juvenile stage. Due to differences in initial weights, two-way ANOVA analysis, initial weights are taken as covariate. Mean values and standard deviations; n=3. *=P<0.001, – P>0.05 (no difference).
Weight gain= Final average weight – Initial average weight †SGR (%/day) = (Ln (final weight (g)) – Ln (initial weight (g)))/(number of days) × 100.

Table 3-9 I Fatty acid composition (% total fatty acids) of livers of juveniles from gilthead sea bream broodstock fed diets with progressive substitution of FO by LO*

Juvenile diet (D)	100FO				80LO				100LO				Two-way ANOVA*		
Broodstock diet (F)	100FO	60LO	80LO	100LO	100FO	60LO	80LO	100LO	100FO	60LO	80LO	100LO			
Total fatty acids (% of dry matter)													D	F	FxD
18:2n-6	2.12±0.68^{B}	1.81±0.49^{B}	1.71±0.14^{B}	1.15±0.07	2.93±0.43^{ABx}	3.19±0.55^{Ax}	2.73±0.26^{Axy}	1.87±0.03^{y}	3.46±0.17^{Ax}	3.97±0.18^{Ax}	3.04±0.23^{Ax}	1.49±0.99^{y}	**	**	-
18:3n-6	0.10±0.02^{B}	0.09±0.02^{B}	0.09±0.00^{B}	0.06±0.00	0.11±0.00^{ABx}	0.11±0.02^{ABx}	0.10±0.01^{ABxy}	0.07±0.01^{y}	0.14±0.01^{A}	0.14±0.00^{A}	0.11±0.01^{A}	0.10±0.04	**	**	-
18:3n-3	0.50±0.19^{C}	0.48±0.17^{C}	0.45±0.06^{C}	0.29±0.05^{B}	3.59±0.42^{By}	3.91±0.53^{Bx}	3.59±0.06^{Bxy}	2.13±0.12^{Az}	4.93±0.59^{Axy}	5.34±0.77^{Ax}	4.20±0.40^{Ay}	3.34±1.06^{Ay}	**	**	*
18:4n-3	0.29±0.08	0.27±0.07^{B}	0.23±0.03	0.15±0.02	0.29±0.02^{xy}	0.31±0.05^{ABx}	0.24±0.03^{xyz}	0.17±0.04^{z}	0.37±0.03^{x}	0.39±0.04^{Ax}	0.29±0.03^{xy}	0.25±0.10^{xy}	**	**	-
20:3n-6	0.05±0.01	0.06±0.02	0.07±0.00	0.04±0.00	0.05±0.01	0.06±0.01	0.07±0.00	0.08±0.07	0.06±0.01	0.07±0.00	0.06±0.01	0.05±0.02	-	-	-
20:4n-3	0.22±0.04	0.25±0.08	0.24±0.01	0.17±0.01	0.20±0.02^{xy}	0.21±0.05^{xy}	0.23±0.01^{x}	0.14±0.01z	0.20±0.02	0.26±0.01	0.21±0.03	0.18±0.07	-	**	-
20:4n-6	0.28±0.05	0.30±0.07	0.29±0.04^{A}	0.26±0.01	0.22±0.02	0.24±0.04	0.22±0.04^{B}	0.18±0.03	0.24±0.05	0.27±0.01	0.17±0.03^{C}	0.19±0.08	**	*	-
20:5n-3	1.73±0.28^{A}	1.91±0.47	1.64±0.04^{A}	1.29±0.10	1.22±0.17^{Bxy}	1.51±0.31^{x}	1.09±0.18^{By}	0.92±0.11^{y}	1.22±0.12^{By}	1.69±0.25^{x}	1.02±0.11^{By}	0.97±0.30^{y}	**	**	-
22:6n-3	3.53±0.61	4.2±1.20	3.85±0.41^{A}	3.24±0.18	2.91±0.22	3.24±0.58	2.88±0.56^{AB}	2.36±0.55	2.84±0.54^{y}	3.64±0.24^{x}	2.35±0.45^{By}	2.38±0.72^{y}	**	*	-

* Mean±SD, n=3 (pool of three livers from each tank), capital superscript letters (A, B) indicate differences between fish fed with different diets during the nutritional challenge coming from the same parental feeding. Low case superscript letters (x,y,z) indicate differences between fish coming from different parental feeding and fed the same diet during the nutritional challenge (P<0.05). All values are mean ± SD (n=3). **=P<0.001, *P<0.01 – P>0.05 (no difference).

Supplemental table 1 | Fatty acid composition (% total fatty acids) of 30 days after hatch larvae obtained from gilthead sea bream broodstock after feeding broodstock diets with progressive substitution of FO*.

	100 FO	40FO 60LO	20FO 80LO†	100LO	*P*
Lipids..	17.60±2.54	18.70±2.10		18.71±1.20	
FA(% of total)					
14:0	0.90±0.07	0.75±0.00		0.81±0.07	*0.107*
14:1n-5	0.33±0.24	0.47±0.02		0.45±0.00	*0.674*
15:1n-5	0.04±0.01	0.04±0.00		0.04±0.00	*0.635*
16:0iso	0.34±0.08	0.33±0.00		0.33±0.01	*0.319*
16:0	16.53±0.59	16.85±0.71		16.00±0.20	*0.741*
16:1n-7	2.13±0.88	1.46±0.01		1.56±0.13	*0.497*
16:1n-5	0.78±0.06	0.72±0.02		0.70±0.01	*0.176*
16:2n-6	0.04±0.00	0.04±0.01		0.05±0.00	*0.302*
16:2n-4	0.16±0.07	0.10±0.04		0.15±0.02	*0.871*
17:0	0.60±0.10	0.60±0.00		0.60±0.00	*0.999*
16:3n-4	0.21±0.08	0.23±0.00		0.23±0.01	*0.902*
16:3n-3	0.07±0.01	0.06±0.01		0.08±0.01	*0.924*
16:3n-1	0.79±0.20	0.75±0.05		0.66±0.02	*0.644*
16:4n-3	0.33±0.03	0.34±0.00		0.32±0.02	*0.735*
16:4n-1	0.20±0.02	0.19±0.00		0.17±0.01	*0.238*
18:0	9.57±0.34	9.29±0.06		9.26±0.07	*0.333*
18:1n-9	15.40±0.89	14.05±1.05		15.61±0.11	*0.614*
18:1n-7	5.80±0.78	5.24±0.31		5.77±0.12	*0.805*
18:1n-5	0.25±0.12	0.16±0.00		0.17±0.02	*0.494*
18:2n-9	0.26±0.03	0.25±0.01		0.26±0.02	*0.966*
18:2n-6	6.32±0.08	6.07±1.30		7.64±0.37	*0.216*
18:2n-4	0.06±0.00	0.06±0.01		0.07±0.01	*0.435*
18:3n-6	0.23±0.05	0.23±0.02		0.25±0.02	*0.856*
18:3n-4	0.05±0.01	0.04±0.01		0.05±0.01	*0.973*
18:3n-3	8.82±3.41	9.02±0.81		9.97±0.28	*0.870*
18:4n-3	1.10±0.44	1.20±0.15		1.28±0.18	*0.229*
18:4n-1	0.03±0.03	0.01±0.00		0.02±0.01	*0.471*
20:0	0.26±0.05	0.23±0.01		0.25±0.00	*0.868*
20:1n-9	0.10±0.06	0.06±0.02		0.09±0.00	*0.796*
20:1n-7	0.87±0.18	0.68±0.13		0.89±0.04	*0.713*
20:1n-5	0.27±0.09	0.20±0.00		0.21±0.01	*0.513*
20:2n-9	0.15±0.12	0.09±0.01		0.08±0.02	*0.594*
20:2n-6	0.43±0.00	0.37±0.06		0.46±0.01	*0.362*
20:3n-9	0.12±0.12	0.08±0.04		0.05±0.03	*0.589*
20:3n-6	0.38±0.13	0.33±0.05		0.29±0.03	*0.531*
20:4n-6	3.07±0.09	3.41±0.28		3.03±0.02	*0.514*
20:3n-3	0.64±0.56	1.01±0.08		1.11±0.03	*0.423*
20:4n-3	0.76±0.11	0.74±0.08		0.80±0.07	*0.865*
20:5n-3	3.69±0.41	3.78±0.07		3.99±0.14	*0.585*
22:1n-11	0.18±0.05	0.15±0.14		0.35±0.00	*0.185*
22:1n-9	0.35±0.12	0.22±0.05		0.29±0.00	*0.513*
22:4n-6	0.11±0.03	0.09±0.00		0.09±0.00	*0.616*
22:5n-6	3.47±0.74	4.23±0.94		3.00±0.15	*0.713*
22:5n-3	0.67±0.12	0.58±0.10		0.67±0.07	*0.971*
22:6n-3	12.65±4.07	14.96±2.37		11.59±0.02	*0.864*
Total FA					
Saturated	28.32±0.99	27.72±0.74		26.91±0.34	*0.473*
n-3	26.73±1.27	31.69±1.07		29.80±0.54	*0.194*
n-6	13.17±0.75	14.76±0.13		14.81±0.17	*0.274*
n-9	17.83±0.84	14.67±1.12		16.34±0.12	*0.531*
n-3 LC-PUFA	16.25±3.66	21.08±2.04		18.16±0.05	*0.852*

* Mean±SD, n=3 (one pool of whole larvae from per tank), no statistical differences were found among the groups (P>0.05).
† No data available.

3.4 Discussion

Given the importance of EPA and DHA for human health (Connor, 2000), and given the limited availability of feedstuffs high in these essential fatty acids (FAO, 2016) , it is necessary to maximize the ability of farmed animals, particularly fish, to synthesize EPA and DHA from their 18C atoms precursors. Among different strategies, nutritional programming of farmed animals to better utilise dietary fatty acids has been previously shown to be very promising (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017). In mammals, nutritional interventions during the early developmental stages through dietary changes along pregnancy and lactation may induce differentiations in phenotypes that are induced by conditions during these stages (Gluckman *et al.*, 2005). Besides, PUFAs are among the nutritional factors known to regulate metabolic decisions of the offspring and adaptation to the environment later in life (Lillycrop and Burdge, 2018).

The results of the present study confirmed that it is possible to induce positive and persistent changes in the LC-PUFA profiles in offspring through parental feeding. Thus, increased replacement of FO by LO in broodstock diets was directly reflected in egg fatty acid profiles, that allowed supplying lower LC-PUFA and higher 18C fatty acid precursors as a nutritional stimulus in an early stage of the development. These changes in eggs fatty acid profiles would be expected, being gilthead sea bream a multi-batch spawner whose oligolectic eggs largely depend on the continuous intake of nutrients during reproduction (Fernández-Palacios *et al.*, 2011). The results are in agreement with the reduced LC-PUFA and increased LA and LNA contents in eggs of gilthead seabream broodstock fed low FO diets found in previous studies (Fernández-Palacios *et al.*, 2011; Izquierdo *et al.*, 2015) (Mourente and Odriozola, 1990) and induced the nutritional programming of the offspring during early embryogenesis. Consequently, during later developmental stages (30 dah larvae), increased FO replacement by LO in the broodstock diets enhanced the offspring ability to synthesise LC-PUFA, as denoted by the increased fatty acids conversion rates and production of end products of fatty acids biosynthesis, DPA and DHA. These adaptations in the offspring fatty acid profiles were

similar when juveniles coming from broodstock fed up to 60% replacement of FO by LO showed increased AA, DHA and, mostly, EPA in the liver when they were fed diets with low LC-PUFA and high 18C fatty acid precursors. These results confirm the long-term effect of feeding broodstock with different FO/LO ratios on the fatty acid profiles of the offspring along life span (Morais *et al.*, 2014; Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017; Adam *et al.*, 2018). However, it is important to note that the long-term effects seems to be related with the ratio between 18C fatty acids especially LA and ALA and n-3 LC-PUFA ratios in the broodstock diets (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017). Studies regarding the effects of 18C fatty acids rich diets are being conducted to give a better understanding of individual fatty acids on nutritional programming of gilthead sea bream (Turkmen et al., in prep).

Changes in larval fatty acid profiles were highly correlated with the up-regulation of *fads2* in seabream larvae coming from parents fed increased FO replacement by LO up to 60%. These results agree well with studies conducted in mammals where maternal diets affect the fatty acid composition of offspring liver, inducing long-term changes in PUFA metabolism with *Fads2* expression (Hoile *et al.*, 2013). The first step of n-3 LC-PUFA synthesis in vertebrates is achieved by fatty acyl delta-6-desaturase (delta-6) enzyme, encoded by *fads2* gene, by introducing a double bond in a specific position of long-chain fatty acids. In the first step of this pathway, LA (18:2n-6) and ALA (18:3n-3) can be converted to 18:3n-6 and 18:4n-3, respectively. After an elongation step, these fatty acids can be converted to 20:3n-6 and 20:4n-3. From these precursors, there is another desaturation step catalysed by a fatty acyl delta-5-desaturase (delta-5) enzyme involved in the conversion to 20:5n-3 (EPA) and 20:4n-6 (AA). Up to date, there is only one desaturase gene (*fads2*) isolated from gilthead sea bream (Seiliez *et al.*, 2003) and low delta-5 activity has been found during *in vitro* studies (Tocher and Ghioni, 1999). Fish species differently evolved and fatty acid desaturases can vary among different species (Castro *et al.*, 2016). For instance, in zebrafish (*Danio rerio*) *fads2* gene gives rise to both delta-5 and delta-6 activities (Hastings *et al.*, 2001). Indeed, in the present study, a strong correlation was found between *fads2* expression levels and conversion rates for

products of delta-6 activity, ALA (18:3n-3) to 18:4n-3 and d5 activity, 20:4n-3 to EPA, from n-3 series. However, such correlations were lower for fatty acids from n-6 series, namely 18:2n-6 (LA) to 18:3n-6 conversion and 20:3n-6 to 20:4n-6. This fact could be related to a higher affinity of *fads2*-derived enzymes to 18:3n-3 and 20:4n-3 substrates rather than 18:2n-6 and 20:3n-6, as proposed for different fish species (Bell *et al.*, 1997; Zheng *et al.*, 2004; Francis *et al.*, 2009; Thanuthong *et al.*, 2011) including gilthead sea bream (Zheng *et al.*, 2004), or to the higher content in ALA in diets and fish tissues. Overall, the present study showed that by modifying the fatty acid profiles of parental diets, namely replacing FO by LO, it is possible to nutritionally program gilthead seabream offspring to be better adapted to utilise low FO/high LO diets during juvenile stages.

However, too large substitution of FO by LO in broodstock diets may have negative consequences for the offspring. For instance, FO replacement of 80 and 100% by LO in broodstock diets lead to reduced fecundity in gilthead seabream, denoted by a reduction in the number of eggs produced, and lower viability of the embryos produced, reflected in a lower survival in 3-dah larvae. These results are in agreement with previous studies (Izquierdo *et al.*, 2015) and are related to the essential role of n-3 LC-PUFA in the embryonic and larval development and the high requirements for these fatty acids in broodstock diets (Fernández-Palacios *et al.*, 1995; Izquierdo *et al.*, 2001). Moreover, these negative effects of too high FO replacement in broodstock diets were persistent along offspring life. Thus, even though all the larvae were fed the same rearing / feeding protocol, there was a lower growth in 30-dah offspring obtained from 100% LO fed broodstock, compared to those from other broodstock groups. Besides, *fads2* expression was showed intermediate level in these larvae although fed with the same commercial diets. It is known that very high replacement of FO by LO in the diets can down-regulate *fads2* expression in gilthead sea bream (Izquierdo *et al.*, 2008; Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017). The mechanisms behind down-regulation of *fads2* by n-3 LC-PUFAs are not clear yet (Tocher, 2003), whereas it is well known that high levels

of n-3 LC-PUFA, especially DHA, can inhibit *fads2* expression in Atlantic salmon (Thomassen *et al.*, 2012; Betancor *et al.*, 2015; Betancor *et al.*, 2016).

Regarding the expression of health related genes, in 30-dah larvae, the increased substitution of FO by LO in broodstock diets up to 60% or 80% respectively lead to the up-regulation of *cox2* or *tnfa*. Cyclooxygenase 2 is one of the enzymes that locally produce in the cells eicosanoids, oxygenated derivatives of LC-PUFA (Rowley *et al.*, 1995), and have a great affinity for AA producing 2-series prostanoids, among them PGE_2 that induces production and release of inflammatory cytokines like TNF-alpha, IL-1 and IL-6 (Calder, 2006). In the present study, up-regulation of *cox2* in 30-dah offspring of broodstock fed 60% LO, despite all larvae were fed the same diet, may be related to the fatty acid profiles in early stages, since eggs from these parents showed the lowest EPA/AA (8.5 in 60% LO vs 9.4 in 100% LO eggs) and, particularly, DHA/AA ratios (18.8 in 60% LO vs 22.0 in 100% LO eggs). Indeed, EPA and DHA reduce gene expression of *Cox2* in mammals (Song *et al.*, 2003; Farooqui *et al.*, 2007) and fish (Ganga, 2010; Torrecillas *et al.*, 2017) and, therefore, lower proportional contents of these fatty acids in developing embryo may have induced the up-regulation of *cox2*, which persisted even in 30-dah larvae. It is possible that, even if *cox2* was up-regulated in offspring from broodstock fed 60%LO, there was a higher EPA and DHA content in eggs that would inhibit the production of PGE_2 avoiding the pro-inflammatory effect of these prostanoids. However, further replacement of FO by LO up to 80% in the diets, led to reduced EPA and DHA contents in the eggs, that could be partly related to the up-regulation of *tnfa*. These results would agree with the up-regulation of *cox2* and *tnfa* found in the posterior gut of seabass juveniles fed reduced dietary levels of n-3 LC-PUFA (Torrecillas *et al.*, 2017). However, expression of other cytokines such as *ilb1*, or histocompatibility complex *mhc1* and *mhc2* were not significantly affected. Still, expression of *mhc2* follows the same trend to be reduced in the offspring with the FO replacement by LO in broodstock diets up to 80% ($R=-0.82$, $P=0.001$). This tendency agrees well with the down-regulation of *mhc2* found in the anterior gut of European sea bass juveniles fed low FM and FO diets (Torrecillas *et al.*, 2017). Nevertheless, the effect of n-3 LC-PUFA

on cytokine production may be different depending on the cellular type, as found in mammals (Petursdottir and Hardardottir, 2007), and it should be considered that in the present study we measured the gene expression levels in the whole body. Overall, these results indicate a significant effect of parental feeding on certain pro-inflammatory genes in the offspring, in agreement with the up-regulation of *tnfa* found also in mammals when parents were fed micronutrients-deficient diets (Kumar *et al.*, 2013). In juveniles of several fish species, FO replacement by VO, namely unbalanced n-3/n-6 ratios, markedly affects stress resistance and plasma cortisol levels (Bell *et al.*, 1991; Ganga, 2010; Montero and Izquierdo, 2010). The heat shock proteins, in turn, facilitate stress response by increasing the binding capacity of the steroid receptor (Basu and Srivastava, 2003). In the present trial, fatty acid profiles of the broodstock diet did not affect *gr* or *hsp90* expression in 30 dah larvae (Izquierdo and Koven, 2011). There was a trend towards up-regulation of *hsp70* with the increased LO up to 80% that would be in agreement with the up-regulation of *hsp70* in European seabass larvae fed increased LA (Izquierdo and Koven, 2011). Further studies must be conducted to clearly determine the potential effects of nutritional programming through broodstock diets on stress resistance of gilthead seabream offspring.

In summary, the present study confirms our previous studies showing that it is possible to nutritionally program gilthead seabream offspring through modification of the fatty acid profiles of parental diets to improve the growth performance of juveniles fed low FO diets, inducing long-term changes in PUFA metabolism and up-regulation of *fads2* expression in larval stage. However, high replacement of FO by LO in broodstock diets may negatively affect embryo and larvae development, reducing growth and survival. Finally, the study also provides the first pieces of evidence of the up-regulation of pro-inflammatory processes related genes in the offspring of seabream fed increased FO replacement by LO. Further studies are being conducted to determine the effects of nutritional programming in stress and disease resistance of seabream offspring, to find the optimal ratios of FO/LO ratio in the parental diets. Other indicators of good performance would also be included in the evaluation.

Author contributions

M.I. and S.T. designed the study, analysed the data and wrote the manuscript. H.F-P., C.M.H. and D.M. conducted broodstock, larvae and juvenile trials, respectively. Molecular biology samples were analysed by S.T. under the supervision of M.J.Z. All authors read and approved the final manuscript. S.T. had primary responsibility for final content. All authors have read and approved the final manuscript.

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Chapter 4

Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream

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Abstract:

The complete removal of fishmeal (FM) and fish oil (FO) is required to promote the sustainable development of aquaculture and for that, fast-growing high-quality fish that are fed without FM and FO are necessary. Early nutritional programming may allow the production of fish better adapted to utilize diets with vegetable meals (VM) and oils (VO). The main objective of this study was to research in the potential value of fatty acids as modulators of early nutritional programming in marine fish for a better utilization of VO/VM. For that purpose, gilthead sea bream (*Sparus aurata*) broodstock were fed four different replacement levels of FO by linseed oil (LO) and their effect on fecundity and spawn quality, egg composition, Δ-6-desaturase (*fads2*) gene expression, progeny growth performance and their growth response to a challenge with diets low in FO and FM, but high in VO and VM. The results showed that feeding gilthead sea bream broodstock with high LO diets had very long-term effects on the progeny. Thus, FO replacement by LO up to 80–100% in broodstock diets for gilthead sea bream not only reduced fecundity and spawn quality, but also growth of 45 dah and 4-month-old juveniles, as well as *fads2* gene expression. However, when the 4-month-old juveniles were fed with a low FM and FO diet, even those from broodstock fed only 60% replacement of FO by LO showed a higher growth and feed utilization than juveniles from parents fed FO. These results demonstrate the interesting potential of early nutritional programming of marine fish by broodstock feeding to improve long-term performance of the progeny. Further studies are being conducted to determine optimum nutrient levels in the broodstock diets and the molecular mechanisms implied to develop effective nutritional intervention strategies for this species.

Statement of relevance:

This study demonstrates for the first time in fish the potential of broodstock nutrition to conduct early nutritional programming of culture fish for a better utilization of low fish meal and fish oil diets by the progeny, showing its effect not only during reproduction and larval development but also during on-growing.

Keywords: HUFA, nutritional programming, broodstock feeding, fishmeal, fish oil, linseed oil

4.1 Introduction

World production of fishmeal (FM) and fish oil (FO) remains stagnant around 6 and 1 million tons, respectively (Kaushik and Troell, 2010). Accordingly, the continuous growth of global aquaculture, a main user of these commodities, requires the efficient use of such high-quality products. Blends of different alternative protein and lipid sources allow substituting FM and FO in diets for salmonids and marine fish (Tacon and Metian, 2008). However, complete or very high replacement levels markedly reduce growth and alter fish metabolism and health (Kaushik *et al.*, 2004; Izquierdo, 2005; Bell and Waagbø, 2008; Torstensen *et al.*, 2008; Montero and Izquierdo, 2010). Therefore, improvements in the utilization of very low FM and FO diets by cultured fish, and the subsequently enhancement of health and growth performance, would be greatly beneficial for the further development of aquaculture. better utilization of low FM and FO diets during on-growing. Very recently, few very promising studies have been conducted in fish to determine the potential of nutritional programming (Vagner *et al.*, 2007; Geurden *et al.*, 2013; Fang *et al.*, 2014) during first exogenous feeding. For instance, the potential long-term metabolic effects of early nutritional programming feeding high carbohydrates diets during first exogenous feeding have been recently studied in zebrafish (*Danio rerio*) (Fang *et al.*, 2014). Nutritional stimulation with high carbohydrates during first feeding affected carbohydrates-related gene expression, digestion, transport and metabolism in the adult fish fed a high carbohydrate diet (Fang *et al.*, 2014), while growth was not affected. Similarly, early short-term feeding of rainbow trout (Oncorhynchus mykiss) fry with a plant-based diet improved acceptance and utilization of the same diet when given at later life stages (Geurden *et al.*, 2013). However, prior to exogenous feeding, during periconception and embryogenesis, vertebrates show a great developmental plasticity, being very sensitive to nutritional changes allowing the organisms the adaptation to adverse postnatal conditions (Duque-Guimaraes and Ozanne, 2013). Nutrition alterations during these early critical periods may permanently influence the organism's metabolism in a process known in mammals as “fetal programming” (Reynolds and Caton, 2012). In fish, embryogenesis and development of most organs mainly occur

before egg hatching and during the so-called "early larval development", before complete depletion of yolk-sac reserves, and is highly dependent on broodstock diet (Izquierdo *et al.*, 2001; Fernández-Palacios *et al.*, 2011). Up to date, no studies have been published on the effect of broodstock feeding on nutritional programming and the progeny performance during on-growing, although the importance of broodstock nutrition for embryogenesis and larval development is well documented (Fernández-Palacios *et al.*, 2011). For instance, elevation of lipid levels in broodstock diets increased both growth and survival of 14 days after hatching (dah) rabbitfish (Siganus guttatus) larvae (Duray *et al.*, 1994) and in several species, increase in n–3 highly unsaturated fatty acids (HUFA), particularly docosahexaenoic acid (DHA), in broodstock diets enhanced growth of first feeding larvae (Fernández-Palacios *et al.*, 1995; Abi-Ayad *et al.*, 1997). However, little is known on the long-term effects of broodstock nutrition on progeny performance during juvenile or adult stages.

Whereas FM contents in on-growing diets can be almost completely replaced by vegetable meal (VM) without any adverse consequence in terms of somatic growth or nitrogen utilization (Kaushik *et al.*, 2004), FO remains to be the key limiting factor for the future growth and sustainability of aquaculture development (De Silva *et al.*, 2011). FO has a unique abundance of n–3 HUFA, important fatty acids derived from linolenic acid that play an essential structural and functional role in fish metabolism. FO replacement relies mainly in vegetable oil (VO), which lack HUFA, but are frequently high in the linolenic acid precursor as it occurs in linseed oil (LO). Freshwater fish generally have sufficient elongase and desaturase activities to produce HUFA from the 18C precursors. On the contrary, marine fish have a very limited capacity to synthesize these fatty acids. Nevertheless, the gene of delta-6-desaturase (*fads2*), the key-limiting enzyme for HUFA synthesis, has been also characterized in marine fish (Vagner and Santigosa, 2011; Li *et al.*, 2014) and its expression can be modulated through the diet (Izquierdo *et al.*, 2008). For instance, in gilthead sea bream (*Sparus aurata*) high levels of dietary FO, rich in HUFA (end products of desaturation and elongation), down-regulated this gene, whereas reduction in FO and increase in LO, rich in the precursor 18:3n– 3, had an up-regulatory

effect (Izquierdo *et al.*, 2008). In one hand, polyunsaturated fatty acids are known to be regulators of gene transcription and expression in fish as in other animals (Benatti *et al.*, 2004; Izquierdo and Koven, 2011) and in the other hand, delivery of nutrients to the embryo from the parental diet can interact with the genome, modify gene expression, and alter protein and metabolite composition in a long-term mode (Feil, 2006). However, it is unknown if fatty acids delivered through the broodstock diet are a suitable nutritional stimulus to modify fatty acid synthesis, the gene expression of key molecular markers such as *fads2* or improve utilization of VO during on-growing. Very few animal and human studies have researched the potential of long chain fatty acids for nutritional programming and most of them have been conducted through first feeding (Bringhenti *et al.*, 2011). However, few studies suggest a role of HUFA on nutritional programming in mammals. For instance, intake of oily fish during pregnancy has been associated with reduced risk of atopic or allergic outcomes in children (Kremmyda *et al.*, 2011).

Therefore, the main objective of this study was to research in the potential value of fatty acids as modulators of early nutritional programming in marine fish for a better utilization of VO. To achieve that aim, gilthead sea bream broodstock were fed four different combinations of FO/LO containing different ratios of HUFA/18:3n – 3 (delta6 products/ precursors). The effect of broodstock feeding on egg composition, *fads2* gene expression, progeny growth performance and their response to a challenge with diets low in FO and FM but high in VO and VM were investigated.

4.2 Materials and Methods

4.2.1 Nutritional stimulus through broodstock diets

Thirty-six brood fish (2–4 years-old) from the gilthead sea bream (S. aurata) broodstock of Grupo de Investigación en Acuicultura (GIA-ULPGC) were randomly selected and distributed in twelve 1000 L fiber-glass tanks. A 2:1 ratio of males to females (Fernández-Palacios *et al.*, 1990) was maintained in each group. At the beginning of the trial, mean body weight and total length for females and males were 1.55 ± 0.40 kg and 1.04 ± 0.29 kg, and 41.25 ± 3.88 cm and 37.2 ± 3.01 cm, respectively

(Table 4-1). Tanks were supplied with 16 L/min filtered seawater (37 ± 0.5‰ salinity) and strong aeration. Seawater temperature during broodstock feeding ranged between 19.41 ± 0.14 and 21.32 ± 0.25 °C, and fish were kept under an indirect natural light (12 h light photoperiod).

Table 4-1 I Biometric characteristics of the gilthead seabream broodstock at the beginning of the feeding trial

Diet	*Body weight (kg)*		*Total length (cm)*	
	*Males**	*Females***	*Males**	*Females***
100FO	1.00±0.20	1.69±0.47	36.95±1.78	42.50±4.54
40FO/60LO	0.99±0.45	1.42±0.40	36.20±4.56	39.40±2.77
20FO/80LO	1.06±0.23	1.39±0.19	37.40±2.79	39.80±2.07
100LO	1.13±0.25	1.79±0.46	38.25±2.27	43.30±4.98

*(mean ± SD, n=6). **(mean ± SD, n=3).

At the beginning of the spawning season, from December 19th to January 15th, fish were fed a commercial diet to ensure that there was no significant differences in the spawning quality of different broodstock, since parent contribution to spawn quality is more important than time (Hamoutene *et al.*, 2009). Afterwards, to conduct the nutritional stimulus trial, from January 16th to June 26th, brood fish were fed one of four experimental extruded diets produced by Biomar.

Table 4-2 I Lipid sources and analysed proximate composition (mean±SD, n=3) of broodstock diets with progressing substitution of fish oil by linseed oil

	Lipid sources (% fresh weight)		*Proximate composition (% dry weight)*			
Diet	*Fish oil STD18*	*Linseed oil*	*Crude protein*	*Crude lipid*	*Moisture*	*Ash*
100FO	8.0	0.0	52.92±0.15	19.51±0.09	7.73±0.07	8.08±0.13
40FO/60LO	3.2	4.8	53.63±0.09	19.08±0.10	8.09±0.09	8.37±0.03
20FO/80LO	1.6	6.4	54.50±0.10	19.13±0.11	7.98±0.12	8.25±0.07
100LO	0.0	8.0	53.29±0.02	19.48±0.10	8.08±0.02	8.00±0.03

The experimental diets were isoproteic and isolipidic (Table 4-2) and their common basal ingredients were 50% fishmeal (SA68 + NALT70), 13.2% sunflower cake, 10% soya cake 48 hi pro solvent extracted, 9.9% wheat, 7% corn gluten 60, and 1.07% vitamin and minerals premix. The diets only differed in their content in fish oil STD18 (FO) and linseed oil (LO): 100% FO, 40%FO/60%LO, 20%FO/ 80%LO and 100%LO (Table 4-2). Substitution of FO in broodstock diets by LO increased total n–3 and n–6 fatty acids (Table 4-3), mainly due to the increase in 18:3n–3 (α-linolenic acid, LNA) and 18:2n–6 (linoleic acid, LA). Besides, LO inclusion reduced saturated, monoenoic and n–3 HUFA fatty acids, in relation to the lower levels of 14:0, 16:0, 16:1n–7, 18:1n–7, 20:1n–9, 20:5n–3 (Eicosapentaenoic acid, EPA) and 22:6n–3 (docosahexaenoic acid, DHA) (Table 4-3). Dietary levels of 18:0, 18:1n–9 and the total sum of n–9 fatty acids were not distinctly affected by the increase in LO. Thus, inclusion of LO raised the 18C precursors of Δ6-desaturase, such as LA and LNA, and reduced the 20 and 22C end products of fatty acid desaturation, such as arachidonic acid (ARA), EPA and DHA.

Table 4-3 | Main fatty acids from total lipids in the broodstock diets with progressing substitution of FO by LO (% total fatty acids)

Fatty acid	100FO	40FO/60LO	20FO/80LO	100LO
14:0	5.90	3.45	2.61	2.69
16:0	17.92	13.48	11.77	11.93
16:1n–7	5.98	3.51	2.73	2.79
18:0	3.11	3.33	3.31	3.37
18:1n–9	15.31	15.72	15.65	16.05
18:1n–7	3.06	2.27	1.97	1.98
18:2n–6	6.46	11.23	13.20	13.13
18:3n–3	1.50	21.36	28.79	28.84
20:1n–9	0.51	0.30	0.23	0.23
20:4n–6	0.63	0.40	0.32	0.30
20:3n–3	0.15	0.11	0.10	0.05
20:4n–3	0.62	0.32	0.23	0.13
20:5n–3	8.79	5.55	4.56	4.13
22:5n–6	0.07	0.04	0.02	0.02
22:6n–3	9.91	6.56	5.32	4.68
Total saturates	27.80	20.81	18.12	18.42
Total monoenoic	37.88	29.94	26.67	27.59
Total n–3	24.85	36.07	40.51	39.38
Total n–6	8.14	12.25	13.93	13.83
Total n–9	16.62	16.53	16.28	16.70
Total n–3HUFA	20.52	13.13	10.60	9.48

Each diet was dispensed to triplicate tanks randomized with respect to dietary treatment. Fish were fed three times a day, a daily ration of 1% initial biomass.

Egg samples of all spawns per tank were collected for biochemical analysis during the initial period fed commercial diet and the second period of feeding the experimental diets.

4.2.2 Spawning and egg quality

Broodstock performance and spawning quality were determined before and after feeding the experimental diets. For that, spontaneously spawned eggs obtained from each experimental broodstock were collected five times per week, during the whole experimental period. Spawning and egg quality were determined by quantifying the total number of eggs and larvae produced, and the proportion of fertilized, viable and hatched out eggs (Fernández-Palacios *et al.*, 2011). Briefly, collected eggs were placed in 5 L beakers provided with aeration, from where 5 randomized 5 mL samples were placed in a Bogorov chamber, counted and observed under binocular microscope to calculate: Total amount of eggs; Percentage of fertilized eggs, determined by the morphological characteristics of the eggs Fernández-Palacios, 2011 #11}; and Egg viability rate, determined as the percentage of morphologically normal eggs at the morula stage and described as transparent, perfectly spherical with clear, symmetrical early cleavages (Fernández-Palacios, 2005). After that, another 5 randomized 5 mL samples were individually placed in a Bogorov chamber, eggs were counted and transferred to five 250 mL crystal beakers filled with sterilized seawater. All the beakers were maintained in a continuous seawater bath to maintain constant temperature. From these samples two different parameters were calculated: Hatching rate and Larval survival at 3 dah (Fernández-Palacios *et al.*, 1995). With these percentages the total numbers of fertilized, viable and hatched eggs and larvae produced per kg female were calculated.

4.2.3 Progeny performance

To determine the effect of the broodstock nutritional stimulus on the progeny, larvae were mass-produced under the intensive conditions similar to those of

commercial farms described here. Eggs collected form each broodstock were distributed in 2000 cylinder-conical tanks (1.5 m diameter and 2.10 m depth) at a density of 125 eggs/L. Sand filtered and UV sterilized seawater flow was progressively increased from 10 to 40% per h until 46 dah. Water was continuously aerated (125 mL/min) attaining 6.1 ± 0.4 ppm dissolved O2. Average water temperature and pH along the trial were 20.8 ± 1.3 °C and 7.89 ± 0.6, respectively. Larvae were reared under a 1500–3500 lx continuous photoperiod (single central light) (Mod TLD 36 W/54, Philips, France) and living phytoplankton (Nannochloropsis sp.) (250 ± 100 × 103 cells per mL) was added to the rearing tanks. From 3–32 dah, larvae were fed twice a day rotifers (Brachionus plicatilis) at 5–10 rot mL^{-1} enriched with commercial emulsions. From 15–17 dah, Artemia enriched with commercial emulsions were added 3 times a day. From 20 dah, larvae were fed commercial diets (Gemma Micro, Skretting, France). At 15, 32 and 46 dah, growth was determined by measuring total length and body weight of 30 anesthetized larvae. At 46 dah, the whole population was transferred to 10,000 L tanks, and fed commercial diets until they reached about 4 g and were ready for the nutritional challenge.

4.2.4 Nutritional challenge of juveniles

To determine the effect of the nutritional stimulus through the broodstock diet on the ability of the progeny to utilize low FO and FM diets, juveniles obtained from broodstock fed the different FO/LO ratios were challenged with two diets either high or low in FM and FO. Juveniles with a mean wet weight of 3.97 ± 0.60 g were randomly distributed into 24 tanks (80 individuals/tank). All tanks (200 L fiberglass cylinder tanks with conical bottom and painted a light gray color) were supplied with filtered seawater (37 ppm salinity) at a rate of 100 L/h to assure good water quality during the entire trial. Water entered from the tank surface and drained from the bottom to maintain high water quality, which were tested daily and no deterioration was observed. Water was continuously aerated (125 mL/min) attaining 6.1 ± 0.3 ppm dissolved O_2. Average water temperature and pH along the trial were 24.6 ± 0.6 °C and 7.89, respectively. Photoperiod was kept at 12 h light:12 h dark by fluorescent daylights and the light

intensity was kept at 1700 lx (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). Diets were formulated and produced by Biomar and they were isoenergetic, one being high in FO (15.30%) and FM (58%) (FOFM diet), and the other extremely low in FO (3%) and FM (5%) (VOVM diet) (Table 4-4). Thus, the FOFM diet had higher levels of ARA, EPA and DHA, whereas the VOVM diet was higher in oleic (18:1n–9), linoleic (18:2n–6), and α-linolenic (18:3n– 3) acids (Table 4-4). Juveniles from each broodstock were daily fed until apparent satiation with one of the two different diets in triplicates for 30 days. Feed consumption was daily recorded. Growth was determined by measuring wet body weight after 24 h starvation.

Table 4-4 I Main ingredients, energy, protein and some fatty acids (% total fatty acids) of diets for the nutritional challenge of gilthead seabream juveniles obtained from broodstock fed different FO and LO ratios

Raw material (%)	FOFM (F)	VOVM (V)
Fishmeal SA 68 Superprime [1]	58.00	5.00
Alternative protein sources [2]	-	54.50
Rapeseed cake	10.00	11.30
Wheat	15.88	6.89
Fish oil SA*	15.30	3.00
Alternative lipid sources**	-	13.00
Fishmeal SA 68 Superprime [1]	58.00	5.00
Alternative protein sources [2]	-	54.50
Biochemical composition (% of dry matter)		
Moisture	10.20	10.96
Protein (crude)	47.80	46.67
Lipids (crude)	21.23	22.45
Ash	10.20	10.96
Energy - gross (MJ kg^{-1})	18.96	18.89
Fatty acids (% of total fatty acids)		
18:1n-9	0.09	0.05
18:2n-6	3.55	19.57
18:3n-3	1.38	11.47
20:4n-6	0.99	0.23
20:5n-3	15.13	3.27
22:6n-3	11.12	2.40

1. South American, Superprime (Feed Service, Bremen, Germany).
2. Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
**Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

Prior to measurements all fish were anesthetized with 10 ppm clove oil:methanol (50:50) in sea water. Whole body weight of all fishes from each tank was determined at 0, 15 and 30 days of feeding.

4.2.5 Biochemical analysis

Diets and eggs were analyzed for proximate and fatty acid composition. Total lipid extraction and quantification, and fatty acid analysis of total lipids were carried out as described previously (Izquierdo *et al.*, 1990).

4.2.6 Molecular studies

At 30 dah, around 100 mg of unfed sea bream larvae obtained from broodstock fed the different FO/LO ratios were collected and conserved in 500 µl of RNA Later (Sigma-Aldrich. Madrid. Spain) overnight at 4ºC, then RNA Later was removed, and samples kept at -80ºC until RNA extraction. Molecular biology analysis was carried out at GIA laboratories. Total RNA from larvae samples (average weight per sample 60mg) was extracted using the RNeasy Mini Kit (Qiagen). Total body tissue was homogenized using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000 G, 15 min, 4 °C). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column where total RNA bonded to a membrane and RW1 and RPE buffers (Qiagen) were used to wash away contaminants. Purified RNA was eluted with 25 µL of RNase-free water. The quality and quantity of RNA were analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:10, 1:100, 1:200 and 1:1000). The product size of the real-time qPCR amplification was checked by electrophoresis analyses using PBR322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using β-actin and RPL27 as house-keeping genes

in a final volume of 20 µL per reaction well, and 100 ng of total RNA reverse transcribed to complementary cDNA. The PCR conditions were the following: 95◦C for 3min 30sec followed by 40 cycles of 95◦C for 15sec, 61◦C for 30sec, and 72◦C for 30sec, 95◦C for 1min., and a final denaturing step from 61◦C to 95◦C for 10sec. Δ6 desaturase expression was determined by real time PCR. Primers for Δ6 desaturase were as follows:

Gen Δ6 desaturase: Forward primers (5'-3') GCAGAGCCACAGCAGCAGGGA, Reverse primers (3'-5') CGGCCTGCGCCTGAGCAGTT

4.2.7 Statistical analysis

The results were expressed as mean ± standard deviation. Data were compared statistically using the analysis of variance (ANOVA), at a significant level of 5%. All variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the Levene tests, respectively (Sokal and Rohlf, 1979). Once significant differences were detected with ANOVA, means were compared by a Tukey test for multiple comparisons of means. A two-way ANOVA was applied to the results of the feeding challenge to determine the combined effect of broodstock and juvenile diets. All data were analyzed using the program STATGRAPHICS (version 5.1 Plus for Windows; Graphic Software Systems Inc., USA).

4.3 Results

4.3.1 Nutritional stimulus through broodstock diets

At the beginning of the spawning season, before the nutritional stimulus, there were no significant differences in the quality of the spawns among the different groups of broodstock. During this period average total number of eggs, fertilized eggs, viable eggs, hatched larvae and 3 day larvae were 69,988 ± 29,152, 57,819 ± 23,560, 54,886 ± 22,541 and 46,030 ± 19,510 per kg female per spawn (mean ± SD). During the nutritional stimulus, increased substitution of dietary FO by LO up to 80-100% LO significantly reduced the total number of eggs produced by kg female per spawn, whereas 60% substitution did not affected this quality parameter (Figure 4-1). The total number of

fertilized eggs followed the same tendency than total eggs (Figure 4-1), since fertilization rates were not affected by the broodstock diet. However, inclusion of LO up to 80-100% markedly reduced the percentage of viable eggs and consequently, total number of viable eggs and hatched larvae were significantly lower when broodstock were fed with these diets than with the 100FO or 40FO/60LO (Figure 4-1). Finally, survival of 3 day larvae was also markedly reduced when broodstock was fed with 20FO/80LO and, particularly, 100LO, and, consequently, a significantly lower number of 3 day larvae (Figure 4-1).

Before the nutritional stimulus through the broodstock diet, egg protein and lipid contents did not significantly differ among the different broodstock (Table 4-5). Egg proximate composition was not significantly affected by feeding the diets with increased LO (Table 4-5). In comparison to the proximate composition of eggs before the study (Table 4-5), after feeding the experimental diets a marked reduction in ash content was noticed in all the treatments, regardless the experimental diet fed to the parents.

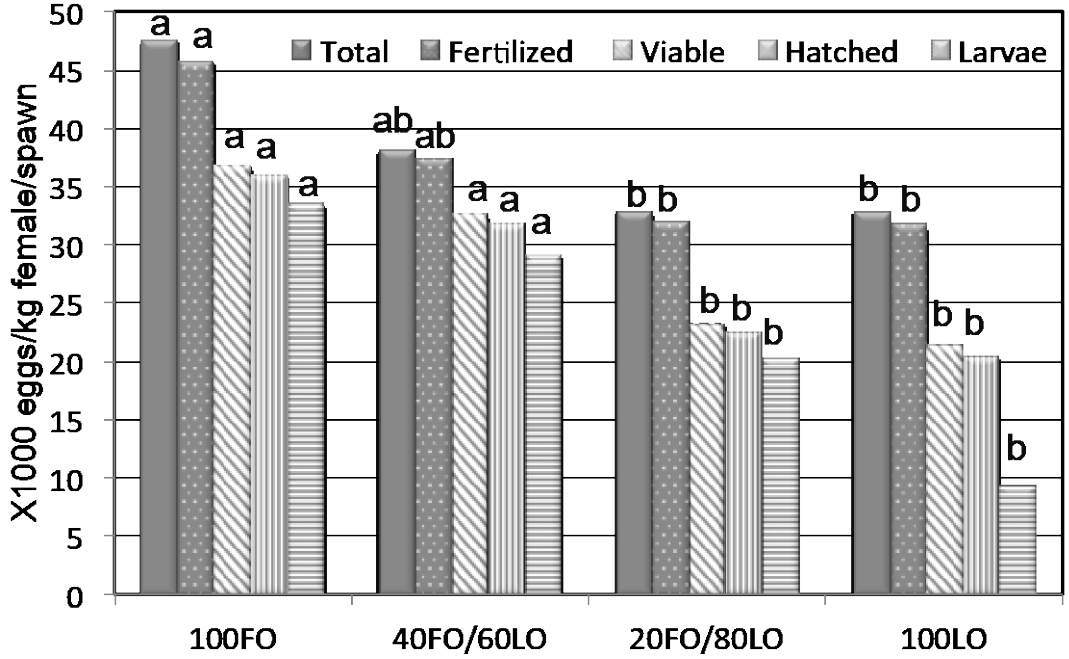

Figure 4-1 | Spawning quality in broodstock fed diets with several proportions of FO and LO during four months of spawning (n= 100; P<0.05).

Table 4-5 | Proximate composition of eggs obtained from gilthead sea bream before and after feeding broodstock diets with progressive substitution of FO by LO (% DM, mean ± SD, n = 3 (one pool of eggs from all the spawns per tank), different superscripts in a column denote significant differences, P<0.05).

	Egg composition before feeding experimental diets			
Diet	**Crude protein**	**Crude lipid**	**Moisture**	**Ash**
100FO	64.08±3.39	24.43±1.11	86.67±0.40^{bc}	15.17±1.39
40FO/60LO	61.44±1.32	23.87±1.69	87.95±0.98^{c}	12.55±2.40
20FO/80LO	60.01±5.31	24.07±0.68	90.52±1.57^{a}	16.90±4.46
100LO	59.87±2.39	23.70±3.77	89.94±1.67^{ab}	16.62±3.68
	Egg composition after feeding experimental diets			
100FO	64.49±4.32	25.55±2.07	87.83±2.16	9.10±4.81
40FO/60LO	71.61±7.91	22.68±2.11	88.16±0.56	9.16±8.11
20FO/80LO	65.82±6.62	25.96±1.71	87.14±0.97	6.09±5.08
100LO	58.34±9.90	21.17±0.63	80.46±2.22	11.92±8.83

Fatty acid composition of the eggs obtained from the broodstock during the adaptation period of four weeks before feeding the experimental diets showed that there were not significant differences among the different broodstock (Table 4-6). In comparison to the egg composition during the adaptation period when fish were fed a commercial diet (Table 4-6), after feeding the experimental diets the level of 20:1n-9,

20:1n-7 and 22:1n-11 was increased (Table 4-7), particularly in FO fish, in relation to the higher monoenoic acid contents in the diet (Table 4-3). Other fatty acids increased in eggs from FO broodstock were the monoens 18:1n-5, 20:1n-9 or 20:1n-7, and other minor fatty acids including 16:3n-3, whereas the level of 18:4n-3 was reduced (Table 4-7). Regardless the diet fed, 18:1n-9, 16:0 and 22:6n-3 were the main fatty acids in gilthead seabream eggs.

FO substitution by LO significantly ($P<0.05$) increased 18:3n-3 and 18:2n-6, particularly in fish fed diets 20FO/80LO and 100LO (Table 4-7), reflecting diet composition (Table 4-3). Besides, regardless dietary inclusion level, LO significantly ($P<0.05$) reduced 16:0 and 17:0 (Table 4-7), lowering the total amount of saturated fatty acids, even despite the levels of 18:0 and 20:0 were not affected. On the contrary, egg levels of monoenoic fatty acids were not so much affected by diet composition. Thus, despite the marked reduction of these fatty acids in the diets with increase content of LO (Table 4-3), eggs from broodstock fed these diets only showed slight reduction in some of these fatty acids such as 16:1n-5, 16:1n-7 or 20:1n-5 (Table 4-7), whereas the other monoens, such as 18:1n-9, 20:1n-9, 20:1n-7 and 22:1n-9, were kept at a similar or higher levels than those of eggs before feeding the experimental diets (Table 4-6).

Despite the high increase of 18:2n-6 in the egg of fish fed increased levels of LO (Table 4-7), other n-6 fatty acids products of desaturation or elongation of this fatty acid, such as 18:3n-6 or 20:2n-6, were not affected or even decreased, such as 20:3n-6, 20:4n-6, 22:4n-6 or 22:5n-6. Regarding the n-3 fatty acids, despite the increase in 18:3n-3 in the egg of fish fed increased levels of LO (Table 4-7), only its direct product of elongation 20:3n-3 was significantly increased, whereas all the other desaturation and elongation products such as 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3 were significantly reduced.

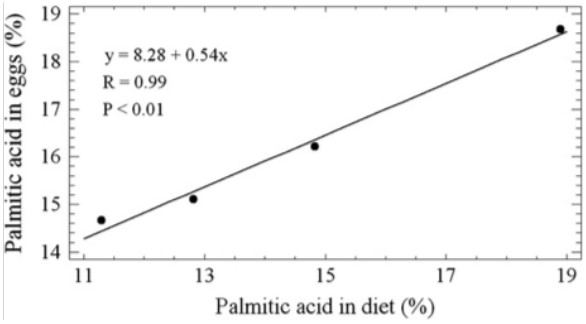

Figure 4-2 | Relation between palmitic acid levels (% total fatty acids) in the broodstock diet and in the eggs of gilthead seabream fed diets with progressive substitution of FO by LO.

Finally, in 40FO/60LO eggs the levels of 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3 and 22:6n-3 were not significantly different than those of FO eggs, while those of 18:2n-6 and 18:3n-3 were significantly ($P<0.05$) lower than those of 20FO/80LO and 100LO eggs (Table 4-7). In general, egg fatty acid profiles were highly correlated with broodstock diet profiles. Thus, linear correlations were significant at $P<0.01$ for 16:0 (Figure 4-2) and 18:1n-7, at $P<0.05$ for 18:3n-3, 18:3n-6, 18:4n-3, 20:1n-7, 20:4n-6, 20:5n-3, 22:6n-3 and 22:5-3 and at $P<0.1$ for 18:2n-6 (data not showed).

Table 4-6 | Fatty acid composition (% total fatty acids) of eggs obtained from gilthead sea bream broodstock before feeding broodstock diets with progressive substitution of FO by LO∗.

Fatty acid	100FO	40FO/60LO	20FO/80LO	100LO
14:0	3.88 ± 0.04	3.78 ± 0.54	3.79 ± 0.18	3.76 ± 0.18
16:0	17.66 ± 0.66	18.75 ± 0.14	18.63 ± 0.33	17.88 ± 0.40
16:1n-7	6.19 ± 0.18	6.58 ± 0.21	6.08 ± 0.48	6.47 ± 0.13
16:1n-5	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.17	0.13 ± 0.01
16:2n-4	0.47 ± 0.04	0.44 ± 0.07	0.44 ± 0.058	0.46 ± 0.07
17:0	0.42 ± 0.04	0.365 ± 0.08	0.370 ± 0.03	0.39 ± 0.07
16:3n-3	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.06	0.11 ± 0.00
16:3n-1	0.07 ± 0.03	0.11 ± 0.07	0.10 ± 0.02	0.10 ± 0.01
16:4n-3	0.26 ± 0.05	0.19 ± 0.04	0.20 ± 0.03	0.22 ± 0.07
18:0	4.08 ± 0.37	5.08 ± 0.80	4.44 ± 0.53	4.21 ± 0.32
18:1n-9	19.23 ± 0.17	18.41 ± 0.68	18.66 ± 0.84	18.51 ± 0.35
18:1n-7	3.11 ± 0.14	3.17 ± 0.03	2.96 ± 0.16	3.04 ± 0.04
18:1n-5	0.11 ± 0.01	0.11 ± 0.00	0.11 ± 0.00	0.11 ± 0.01
18:2n-9	0.16 ± 0.02	0.14 ± 0.04	0.14 ± 0.03	0.17 ± 0.02
18:2n-6	8.35 ± 0.41	7.93 ± 0.25	8.05 ± 0.46	7.86 ± 0.36
18:3n-6	0.28 ± 0.04	0.36 ± 0.03	0.26 ± 0.05	0.28 ± 0.04
18:3n-3	2.74 ± 1.12	2.60 ± 0.74	2.65 ± 1.07	2.29 ± 0.75
18:4n-3	0.82 ± 0.044	0.77 ± 0.06	0.71 ± 0.09	0.78 ± 0.12
20:0	0.09 ± 0.02	0.10 ± 0.04	0.09 ± 0.01	0.09 ± 0.03
20:1n-9	0.09 ± 0.00	0.13 ± 0.05	0.08 ± 0.01	0.08 ± 0.01
20:1n-7	0.60 ± 0.00	0.58 ± 0.00	0.55 ± 0.01	0.53 ± 0.04
20:1n-5	0.15 ± 0.01	0.18 ± 0.02	0.143 ± 0.02	0.14 ± 0.02
20:2n-9	0.08 ± 0.01	0.08 ± 0.00	0.07 ± 0.02	0.08 ± 0.02
20:2n-6	0.28 ± 0.01	0.22 ± 0.04	0.26 ± 0.03	0.236 ± 0.01
20:3n-9	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
20:3n-6	0.17 ± 0.02	0.19 ± 0.01	0.16 ± 0.03	0.15 ± 0.01
20:4n-6	0.91 ± 0.06	0.88 ± 0.08	0.95 ± 0.092	0.93 ± 0.02
20:3n-3	0.25 ± 0.08	0.20 ± 0.03	0.25 ± 0.09	0.19 ± 0.04
20:4n-3	0.77 ± 0.07	0.77 ± 0.05	0.69 ± 0.07	0.72 ± 0.07
20:5n-3	7.30 ± 0.13	7.49 ± 0.16	7.09 ± 0.27	7.55 ± 0.77
22:1n-11	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.09 ± 0.01
22:1n-9	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.07 ± 0.01
22:4n-6	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
22:5n-6	0.19 ± 0.010	0.17 ± 0.007	0.18 ± 0.02	0.18 ± 0.01
22:5n-3	2.99 ± 0.09	2.81 ± 0.13	2.84 ± 0.22	2.95 ± 0.15
22:6n-3	16.21 ± 1.24	15.23 ± 0.76	16.89 ± 1.58	17.55 ± 0.72

Mean ± SD, n = 3 (one pool of eggs from all the spawnings per tank), different superscripts for each fatty acid would denote significant differences, P<0.01.

Table 4-7 | Fatty acid composition (% total fatty acids) of eggs obtained from gilthead sea bream broodstock after feeding broodstock diets with progressive substitution of FO∗.

Fatty acid	100FO	40FO/60LO	20FO/80LO	100LO
14:0	4.43 ± 0.57	2.78 ± 0.34	1.369 ± 1.50	1.59 ± 1.36
16:0	18.68 ± 0.08a	16.21 ± 0.56b	15.11 ± 0.90b	14.66 ± 0.95b
16:1n–7	5.91 ± 0.65a	4.15 ± 0.25b	3.68 ± 0.34b	3.57 ± 0.25b
16:1n–5	0.13 ± 0.01a	0.09 ± 0.01b	0.08 ± 0.01b	0.06 ± 0.02b
16:2n–4	0.31 ± 0.03a	0.20 ± 0.02b	0.20 ± 0.21b	0.19 ± 0.01b
17:0	0.23 ± 0.02a	0.16 ± 0.01b	0.15 ± 0.02b	0.14 ± 0.01b
16:3n–3	0.16 ± 0.01a	0.11 ± 0.01b	0.09 ± 0.01b	0.08 ± 0.01b
16:3n–1	0.12 ± 0.02	0.11 ± 0.02	0.12 ± 0.1	0.10 ± 0.01
16:4n–3	0.17 ± 0.02a	0.07 ± 0.05b	0.08 ± 0.02b	0.07 ± 0.03b
18:00	3.71 ± 0.14	4.21 ± 0.63	4.34 ± 0.07	460 ± 0.157
18:1n–9	18.18 ± 0.88	16.93 ± 0.51	17.87 ± 0.30	17.99 ± 0.79
18:1n–7	3.08 ± 0.14a	2.41 ± 0.08b	2.24 ± 0.07b	2.11 ± 0.11b
18:1n–5	0.22 ± 0.02a	0.15 ± 0.01b	0.13 ± 0.02b	0.11 ± 0.01b
18:2n–9	0.12 ± 0.06	0.11 ± 0.03	0.08 ± 0.02	0.09 ± 0.02
18:2n–6	7.01 ± 0.56c	10.46 ± 0.84b	12.60 ± 0.42a	13.46 ± 0.41a
18:3n–6	0.25 ± 0.08	0.25 ± 0.03	0.23 ± 0.04	0.21 ± 0.03
18:3n–3	2.24 ± 0.17c	13.08 ± 1.41b	18.87 ± 2.57a	21.23 ± 1.39a
18:4n–3	1.23 ± 0.11a	0.742 ± 0.01b	0.69 ± 0.04b	0.56 ± 0.10b
20:00	0.13 ± 0.01	0.14 ± 0.03	0.14 ± 0.03	0.13 ± 0.03
20:1n–9	0.30 ± 0.04a	0.16 ± 0.01b	0.18 ± 0.04b	0.12 ± 0.01b
20:1n–7	1.40 ± 0.23a	0.86 ± 0.01b	0.83 ± 0.14b	0.685 ± 0.03b
20:1n–5	0.17 ± 0.01a	0.12 ± 0.01b	0.12 ± 0.01bc	0.10 ± 0.01c
20:2n–9	0.06 ± 0.13a	0.04 ± 0.01ab	0.03 ± 0.01ab	0.03 ± 0.03b
20:2n–6	0.24 ± 0.02	0.26 ± 0.01	0.29 ± 0.03	0.25 ± 0.01
20:3n–9	0.04 ± 0.00a	0.03 ± 0.02b	0.02 ± 0.01c	0.02 ± 0.01c
20:3n–6	0.11 ± 0.01a	0.10 ± 0.01ab	0.07 ± 0.01b	0.06 ± 0.01b
20:4n–6	0.79 ± 0.07a	0.67 ± 0.09ab	0.51 ± 0.06bc	0.48 ± 0.04c
20:3n–3	0.16 ± 0.00c	0.50 ± 0.04b	0.84 ± 0.11a	0.71 ± 0.04a
20:4n–3	0.83 ± 0.11a	0.53 ± 0.02b	0.42 ± 0.07bc	0.31 ± 0.04c
20:5n–3	7.05 ± 0.86a	5.43 ± 0.41ab	4.12 ± 0.3bc	3.626 ± 0.23c
22:1n–11	0.60 ± 0.10a	0.29 ± 0.00b	0.25 ± 0.05b	0.19 ± 0.02b
22:1n–9	0.15 ± 0.01	0.11 ± 0.01	0.13 ± 0.05	0.08 ± 0.01
22:4n–6	0.07 ± 0.03a	0.06 ± 0.01ab	0.04 ± 0.01bc	0.03 ± 0.00c
22:5n–6	0.22 ± 0.02a	0.16 ± 0.02b	0.12 ± 0.01bc	0.09 ± 0.01c
22:5n–3	2.21 ± 0.29a	1.93 ± 0.13ab	1.47 ± 0.30b	1.33 ± 0.15b
22:6n–3	17.55 ± 1.47a	15.09 ± 1.47a	10.98 ± 1.25b	10.24 ± 0.85b

Mean ± SD, n = 3 (one pool of eggs from all the spawnings per tank), different su- perscripts for each fatty acid would denote significant differences, $P<0.01$.

4.3.2 Progenie performance

At 15 or 32 dah, larval growth in terms of total length or body weight was not significantly affected by the nutritional stimulus trough the broodstock diets (Table 4-8). However, at 46 dah, despite all larvae were fed the same commercial protocol of rotifers, Artemia and weaning diets, those obtained from broodstock fed 100% FO (100FO larvae) showed the highest ($P<0.01$) total length and body weight, followed by 40FO/60LO larvae (Table 4-8). The significantly ($P<0.01$) lowest total length and body weight were obtained in 100L larvae, without significant differences with 20FO/80LO larvae. Regarding the molecular studies, parental feeding with increased LO up to 20FO/80FO, progressively increased relative expression of Δ6 desaturase gene in the 30 dah progenie (Figure 4-3). On the contrary, complete FO replacement with LO significantly down-regulated Δ6 desaturase gene expression (Figure 4-3).

Table 4-8 | Growth (mean ± SD) of larvae obtained from broodstock fed diets with progressive substitution of FO by LO and fed the same commercial feeding protocol from first feeding.

	Larval age	Parental Diet			
		100FO	40FO/60LO	20FO/80LO	100LO
Total length (mm)	15dah	5.44±0.41	5.11±0.39	5.26±0.4	5.20±0.37
	32dah	8.81±1.24	8.61±1.51	7.96±1.25	8.84±1.4
	46dah	13.16±1.26^{a}	11.99±0.95^{b}	11.23±1.03^{c}	11.14±1.04^{c}
Body weight (mg)	15dah	1.12±0.07	1.00±0.139	0.97±0.037	0.91±0.054
	32dah	5.87±0.41	5.39±0.859	4.20±0.195	5.08±0.155
	46dah	14.88±3.94^{a}	9.87±1.93^{b}	7.89±2.88^{bc}	7.34±1.37^{c}

*n = 30, **n = 9. Different low-case letters at a given day denote significant differences ($P<0.01$).

After 4 months of rearing, survival from hatching of 100FO, 40FO/60LO, 20FO/80LO and 100 L juveniles was, respectively, 15.07, 13.44, 12.01, 11.72%, whereas average body weight reached 4.44, 3.37, 3.67 and 3.35g.

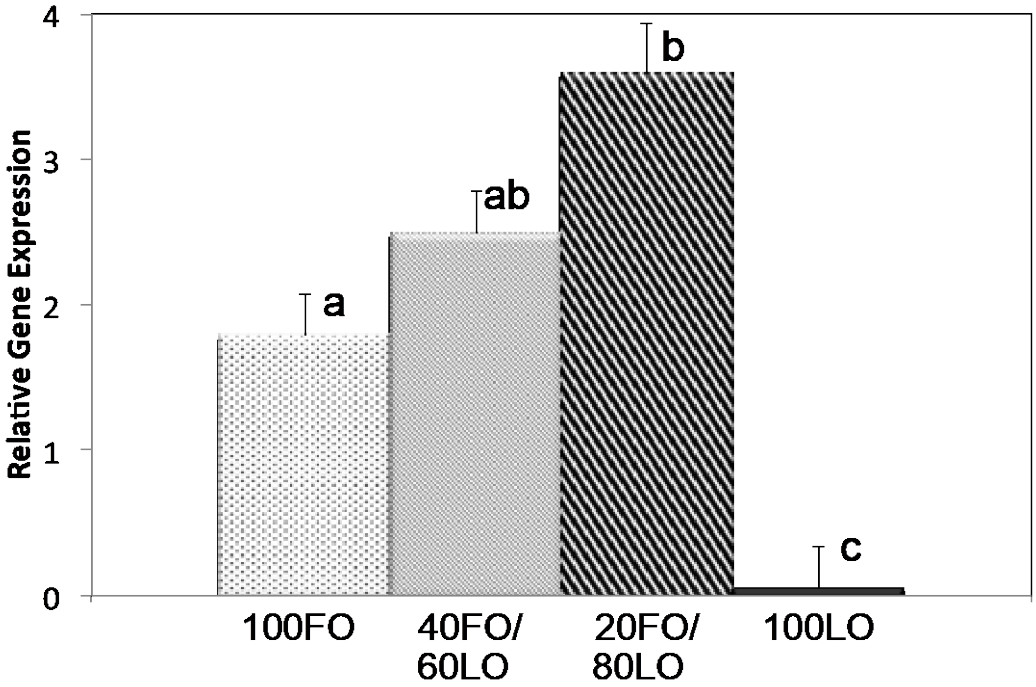

Figure 4-3 | Relative expression of Δ6 desaturase gene in 30 dah progeny obtained from broodstock fed diets with progressive substitution of FO by LO (n = 3, P<0.05).

4.3.3 Nutritional challenge of juveniles

All the experimental diets were well accepted by gilthead seabream juveniles during the entire trial. No significant differences were observed in feed intake between triplicates for each diet and group.

Before the nutritional challenge there were no significant differences between initial weights of the different experimental groups (P>0.05), whereas after only 15 days of feeding significant differences were found in growth among fish fed the VOVM diet (Figure 4-4). Thus, increase in the LO content in the broodstock diet promoted SGR in juveniles fed VOVM diets (Figure 4-4). After 30 days of feeding, fish fed the FOFM diet showed significantly (P<0.001) higher body weights than those fish fed VOVM diet (Table 4-9). More interestingly, after 30 days of feeding there was an effect of the parental feeding. Thus, although all juveniles performed equally well under a FOFM diet, among fish fed the VOVM diet those coming from the parents fed higher LO levels, showed a significantly (P<0.05) higher growth. Hence, fish from broodstock fed 100FO had a lower

final weight than 40FO/60LO and 20FO/8LO, which in turn were lower than 100LO (Table 4-9). The same trend was observed for SGR, which was significantly (P<0.001) higher for fish fed diet FOFM than for those fed VOVM. No significant differences were found in SGR for fish fed the FOFM diet, while among the fish fed VOVM those coming from broodstock fed higher LO showed higher SGR (P<0.05). Besides, the diet had a pronounced effect on feed conversion ratio (FCR) and fish fed FOFM had lower FCR than those fed VOVM diet (P b 0.001). Moreover, FCR was not different among groups fed the FOFM diet, but when fish were fed the VOVM diet those from broodstock fed 100FO showed the highest FCR (P<0.01). Thus, the two-way ANOVA showed a significant effect of the type of the juvenile diet and the broodstock diet on body weight, SGR and FCR and a combined effect of both broodstock and juvenile diets on FCR.

Table 4-9 I Effect of nutritional challenge with high (FOFM) or low (VOVM) fishmeal and fish oil diets on growth and feed utilization of gilthead sea bream (Sparus aurata) juveniles obtained from broodstock fed diets with progressive substitution of FO by LO (mean ± SD, n = 3)*.

Juveniles diet	Broodstock diet	Body weight (g)		SGR[1] (%BW/day)	FCR[2]
		Initial	Day 30		
FOFM	*100FO*	4.01±0.59	^{A}8.96±1.91^{ab}	^{A}2.67±0.12	^{A}1.26±0.15
	40FO/60LO	4.01±0.58	^{A}8.94±1.78^{ab}	^{A}2.73±0.25	^{A}1.30±0.01
	20FO/80LO	3.94±0.61	^{A}8.64±1.40^{b}	^{A}2.45±0.07	^{A}1.37±0.15
	100LO	3.93±0.59	^{A}9.31±1.69^{a}	^{A}2.87±0.06	^{A}1.33±0.06
VOVM	*100FO*	4.03±0.59	^{B}6.88±1.50^{c}	^{B}1.77±0.23^{b}	^{B}2.07±0.15^{b}
	40FO/60LO	3.92±0.62	^{B}7.35±1.31^{b}	^{B}2.10±0.17^{ab}	^{B}1.57±0.06^{a}
	20FO/80LO	3.97±0.62	^{B}7.29±1.43^{b}	^{B}2.03±0.11^{ab}	^{B}1.63±0.12^{a}
	100LO	3.95±0.63	^{B}7.73±1.44^{a}	^{B}2.23±0.15^{a}	^{B}1.60±0.01^{a}
Two-way ANOVA*					
Juveniles diet		n.d.	P<0.001	P<0.001	P<0.001
Broodstock diet		n.d.	P<0.001	P<0.05	P<0.01
Juveniles diet × Broodstock diet		n.d.	P<0.01	n.d.	P<0.01

* Superscripts with different high-case letters in a column denote significant differences between juveniles' dietary treatments and different low-case letters denote significant differences among juveniles fed the same diet but coming from different broodstock. SGR (specific growth rate) = [Ln (final body weight, g) – Ln (initial body weight, g)] / (experimental period in days) × 100. FCR (feed conversion ratio) = (total weight of consumed feed, g) / (weight gain, g).

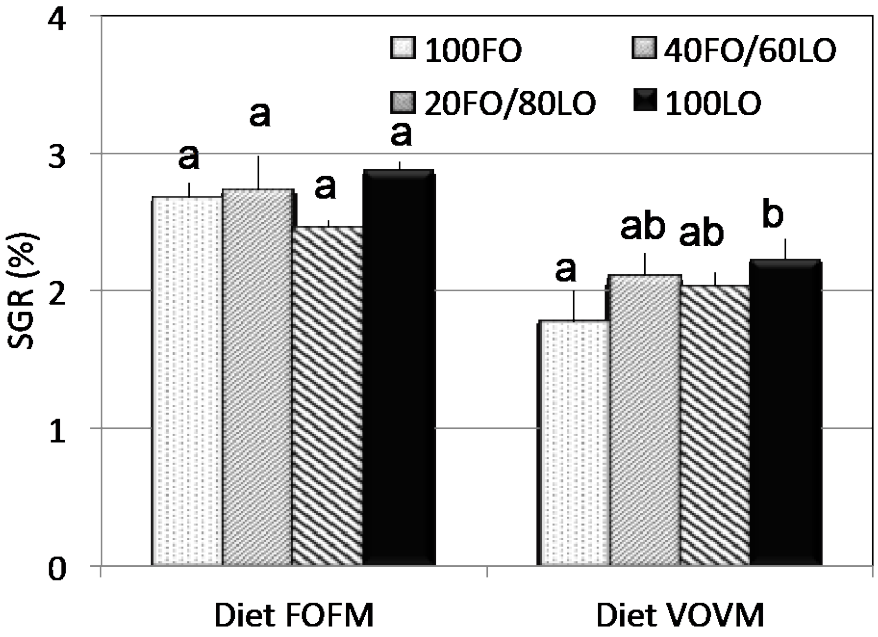

Figure 4-4 I Specific growth rate (SGR) of gilthead sea bream juveniles from broodstock fed diets with progressive substitution of FO by LO after 15 days of a nutritional challenge with FOFM or VOVM diets (n = 3, $P < 0.05$). Letters indicate significant differences among juve- niles fed the same diet. SGR = [Ln (final body weight, g) – Ln (initial body weight, g)] / (experimental period in days) × 100.

4.4 Discussion

Dependency on dietary ingredients with a limitation in constant supply such as FO or FM may constrain the further development of aquaculture. Therefore, any progress in the utilization of low FM and FO diets by cultured fish will contribute for the sustainable development of aquaculture. The present study investigated how an early nutritional stimulus of the developing embryo through the broodstock diet may affect the performance of the progeny, particularly when juveniles are fed low FM and FO diets.

The results demonstrated the profound effect of broodstock feeding on the later performance of the progeny. A large (80%) substitution of FO by LO in broodstock diets and, hence, an increase in dietary linolenic acid and a reduction in HUFA, markedly reduced the total number of eggs produced, denoting a negative effect on gametogenesis. Indeed HUFA-derived prostaglandins and leukotrienes synthesized within the ovary control ovulation and oviposition (Fernández-Palacios *et al.*, 2011). Fatty acid profiles of eggs from broodstock before feeding the experimental diets did not differ

significantly, in agreement with the high spawning quality in all brood fish during this period. However, feeding increased LO levels markedly reduced HUFA contents in the eggs and the number of larvae obtained. In sparids, the fatty acid composition of the female gonad is greatly affected by the dietary fatty acid content (Izquierdo *et al.*, 2001), and, thus, the reduction in the number of hatched and 3 dah larvae denoted the importance of HUFA during embryogenesis and larval development. In fish larvae, these fatty acids have an important structural function as components of the phospholipids in the fish bio-membranes (Izquierdo *et al.*, 2000). They are also a source of metabolic energy for the embryo, precursors of eicosanoids that regulate cell proliferation and differentiation and regulators of gene transcription and expression (Izquierdo and Koven, 2011).

In agreement with former studies in this and other species (Fernández-Palacios *et al.*, 1995; Izquierdo, 1996), 18:1n–9, 16:0 and 22:6n– 3 were the main fatty acids in gilthead sea bream eggs. The ratio between saturated and unsaturated fatty acids regulates the fluidity of cell and organelle membranes and thereby their function (Sargent *et al.*, 1999). Palmitic acid (16:0) was markedly reduced by the inclusion of LO in broodstock diets, despite being a major fatty acid in gilthead sea bream eggs, an important constituent fatty acid of fish phospholipids and potentially synthesized by fish. Given the importance of this fatty acid in gilthead sea bream eggs and the high dependence on the dietary levels, the palmitic acid content in low FO broodstock diets should be balanced by the inclusion of VO rich in this fatty acid, such as palm oil. In general, inclusion of palm oil up to 10–15% can effectively replace FO without affecting fish performance (Ng and Gibon, 2010). The high correlation of egg fatty acid profiles with broodstock diet fatty acids denotes the high dependency of the vitelogenetic process on dietary lipids rather than in body lipids in gilthead sea bream, allowing in this species to conduct an effective early nutritional intervention on the embryo through the broodstock diet. Interestingly, LO inclusion up to 60% substitution of FO, although moderately modifying fatty acid profiles, did not significantly affect spawn quality, what

may allowed to exert a nutritional stimulus of the embryo without negatively affecting its development.

Growth of larvae from parents fed different proportions of FO and LO was very similar in body weight and total length at 15 and 30 dah. How- ever, increase in broodstock diet LO from 0 to 60, 80 and 100% was reflected in a 8, 15 and 15% reduction in total length in 45 dah larvae (almost post-metamorphosis) and a 22% lower body weight in 3–4 g juveniles. In agreement, in mammals, nutritional programing through parental diet caused changes in the adults' weight, metabolism and immune responses, even if offspring size at birth was not affected (Sinclair *et al.*, 2007). Alterations in the progeny metabolism occurred as well in the present study as denoted by the up-regulation of *fads2* gene expression in 15 dah larvae by increased LO up to 80% replacement in the broodstock diets, despite from first feeding all the larvae were fed the same commercial protocol. Moreover, complete replacement of FO (rich in HUFA) by LO (rich in 18:3n– 3) inhibited the expression of this gene, in agreement with the down-regulation found by first- feeding gilthead sea bream larvae fed 100% LO (Izquierdo *et al.*, 2008). In other marine fish species, the *fads2* transcript levels in liver of fish fed VO were up-regulated in comparison to those fed FO, whereas complete replacement of FO by LO and perilla oil down-regulated this gene (Xu *et al.*, 2014). Thus, *fads2* may be considered an effective key biological marker of essential HUFA synthesis and the ability of marine fish to utilize VO. In fish, as in other animals, HUFA function as ligands involved in gene transcription and expression, regulating a broad series of genes (Izquierdo and Koven, 2011; Izquierdo *et al.*, 2013; Saleh *et al.*, 2014). Thus, preliminary studies feeding gilthead sea bream broodstock with high LO (Izquierdo *et al.*, 2013) or alpha-tocopherol diets (Turkmen et al., accepted) have also demonstrated the alteration in the expression of several genes in the larvae, such as TNF-alfa, GR or MCH-2 in 30 dah larvae. In mammals, parental feeding micronutrients-deficient diets led to low levels of interleukin-1b (IL-1b) and high levels of tumor necrosis factor a (TNF-a), which were related to and an increased inflammatory state that could contribute to metabolic alterations in later life (Kumar et al., 2013).

Increased LO in broodstock diets lead to growth reduction in gilthead sea bream larvae and juveniles reared with commercial diets, but when juveniles were challenged with an extremely low FM (5%) and FO (3%) diet parental feeding with LO lead to a significant improve- ment in SGR, FCR and final body weight. Feeding marine fish with very low FM or FO diets has been found to markedly reduce fish growth (Glencross *et al.*, 2003; Almaida-Pagan *et al.*, 2007; Benedito-Palos *et al.*, 2007; Montero *et al.*, 2008). In the present study FM and FO substitution levels were even higher (91% FM substitution and 80% FO substitution) than the levels tested by those authors (60 and 80% substitution). Under such unfavorable nutritional conditions, fish whose parents were fed with low FO diets performed significantly better. These results are in agreement with the higher growth of gilthead sea bream juveniles fed a high LO diet when broodstock was fed LO (Izquierdo *et al.*, 2013). The present study demonstrated that, as it occurs in other animals (Burdge and Lillycrop, 2010), the early life environment plays a commanding role in adult onset of metabolic alterations and, therefore, interventions during this critical period might provide opportunities to modulate metabolism later in life if fish confront again those sub-optimal conditions. Although this is the first study in fish that demonstrates the alteration of the progeny performance and metabolism by the modulation of the broodstock diet, in different animal models the periconceptual nutritional status has been found to alter offspring metabolism (Todd *et al.*, 2009). Nevertheless, most of the studies conducted to determine the effect of prenatal nutritional environment on the ultimate phenotype of the organism have studied maternal or paternal diet focusing mainly in low protein diets, global caloric restriction or micronutrient deficiency (Duque-Guimaraes and Ozanne, 2013). Only very few studies in mam- mals have demonstrated that a maternal high n–6 fatty acid diet results in a greater risk of breast cancer in the female offspring (Su *et al.*, 2011). The present study demonstrates the effect of fatty acid profiles in parental diets on the long-term growth performance of the progeny. Further studies to explain the molecular and metabolic response in key tissues of these juveniles reared under high (FMFO diets) or low-class (VMVO diets) environments have been also conducted (Turkmen et al., in press).

Inclusion of LO in gilthead sea bream broodstock improved the performance of juveniles challenged with a low FM and FO diet, but the two most extreme levels had a negative impact in fecundity, spawn quality and larval growth than the 60% substitution level. Therefore, further studies will be conducted to determine the optimum LO inclusion level in broodstock diets to improve low FM and FO diet utilization along on-growing, without affecting broodstock, larvae or juvenile performance.

In conclusion, the results of this study have shown the long-term effects of feeding gilthead sea bream broodstock with high LO diets. Thus, FO replacement by LO up to 80–100% in broodstock diets for gilthead sea bream not only markedly reduce fecundity and spawn quality, but also the size of 45 dah and 4-month-old juveniles, as well as the *fads2* ex-pression. However, even only 60% replacement of FO by LO produced juveniles with a better ability to utilize low FM and FO diets and a higher growth, demonstrating the interesting potential of early nutritional programming of marine fish by broodstock feeding to improve long-term performance of the progeny. Further studies are being conducted to determine optimum levels and the molecular mechanisms implied to develop effective nutritional intervention strategies for this species.

Chapter 5

Parental nutritional programming and a reminder during the juvenile stage affect growth, lipid metabolism and utulisation in later developmental stages of a marine teleost, the gilthead sea bream

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Abstract:

Nutrition during periconception and early development can modulate metabolic routes to prepare the offspring for adverse conditions through a process known as nutritional programming. In gilthead sea bream, replacement of fish oil (FO) with linseed oil (LO) in broodstock diets improves growth in the 4-month-old offspring challenged with low-FO and low-fishmeal (FM) diets for 1 month. The present study further investigated the effects of broodstock feeding on the same offspring when they were 16 months old and were challenged for a second time with the low-FM and low-FO diet for 2 months. The results showed that replacement of parental moderate-FO feeding with LO, combined with juvenile feeding at 4 months old with low-FM and low-FO diets, significantly ($P<0.05$) improved offspring growth and feed utilisation of low-FM/FO diets even when they were 16 months old: that is, when they were on the verge of their first reproductive season. Liver fatty acid composition was significantly affected by broodstock or reminder diets as well as by their interaction. Moreover, the reduction of long-chain PUFA and increase in α-linolenic acid and linoleic acid in broodstock diets lead to a significant down-regulation of hepatic lipoprotein lipase ($P < 0{\cdot}001$) and elongation of very long-chain fatty acids protein 6 ($P<0.01$). Besides, fatty acid desaturase 2 values were positively correlated to hepatic levels of 18:4n-3, 18:3n-6, 20:5n-3, 22:6n-3 and 22:5n-6. Thus, this study demonstrated the long-term nutritional programming of gilthead sea bream through broodstock feeding, the effect of feeding a 'reminder' diet during juvenile stages to improve utilisation of low-FM/FO diets and fish growth as well as the regulation of gene expression along the fish's life-cycle.

Keywords: nutritional adaptation of offspring, long term effects of parental nutrition, hepatic gene expression, fatty acid desaturase (*fads2*), epigenetics in aquaculture

5.1 Introduction

Aquaculture is the fastest growing animal production sector accounting at present more than 50% of the world consumed fish (FAO, 2016), but one major issue concerning such development is the over dependence of fish feeds on capture fishery-derived raw materials such as fishmeal (FM) and fish oil (FO) (Kaushik and Troell, 2010). The established beneficial roles of FO on human health (Wang *et al.*, 2006) and the use of FO, albeit in small proportions, in other animal production systems, have led to an increase in demand for this raw material, consequently raising the prices. Despite the great achievements to reduce FM in the diets of marine fish species (Kaushik *et al.*, 2004; Benedito-Palos *et al.*, 2007; Le Boucher *et al.*, 2011; Le Boucher *et al.*, 2013; Shepherd and Bachis, 2014), complete replacement of FO remains still a major challenge. Moreover, complete substitution of FO negatively affects immune system and stress and disease resistance (Montero and Izquierdo, 2010) and reduces the fillet content in long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA), includes 20 or carbon atoms), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), negatively affecting the nutritional value of fish flesh for humans (Izquierdo *et al.*, 2005; Rosenlund *et al.*, 2010; Eroldoğan *et al.*, 2013; Yılmaz *et al.*, 2016).

FO is rich in n-3 LC-PUFA whereas vegetable oil (VO) sources, except in some cases(Betancor *et al.*, 2015), lack the essential fatty acids (EFA) for marine fish such as EPA and DHA but can have significant amounts of alpha-linolenic acid, 18:3n-3 (ALA) and linoleic acid, 18:2n-6 (LA) biological precursors of EFA. Yet bioconversion of 18 carbon PUFAs to EPA and DHA depends on the elongation and desaturation capacity of the fish species (Sargent *et al.*, 2002; Castro *et al.*, 2016). Generally speaking, whereas freshwater fish possess the ability to convert ALA and LA into LC-PUFA (Sargent *et al.*, 1999; Leaver *et al.*, 2008), marine fish do not possess the sufficient enzyme activity (Tocher, 2010). Nonetheless, LC-PUFA synthesis capacity also appears to differ among marine species (Monroig *et al.*, 2012; Monroig *et al.*, 2013; Navarro-Guillén *et al.*, 2014; Xu *et al.*, 2014). The higher LC-PUFA biosynthesis capacity in freshwater fish in

comparison to marine fish could be related to differences in the feeding habits and nutrients intake, with marine fish having a continuous access to LC-PUFA rich sources throughout their lives (Sargent *et al.*, 1995; Tocher, 2003). Besides, these differences among fish species have been related to the diverse evolution of certain genes involved in lipid biosynthesis (Castro *et al.*, 2016).

Recent evidence suggests that environmental factors experienced by the parents can have long-lasting effects in the offspring or in the later generations (Burdge *et al.*, 2007; Burton and Metcalfe, 2014). Thus, early environmental signs, such as available nutrients during reproduction, can modulate metabolic routes and offspring phenotype (Gluckman and Hanson, 2004; Burdge *et al.*, 2007; Curley *et al.*, 2011). This type of metabolic regulation, known as "nutritional programming" has been principally derived from mammalian models, due their potential effects on development of metabolic disorders in humans in later life (Langley-Evans, 2015). Therefore, better understanding of outcomes parental nutrition and the underlying mechanisms can contribute to the prevention of consequences in offspring. Besides, nutritional programming may also have potential applications in animal production (Gotoh, 2015). In aquaculture, one of the potential beneficial applications of nutritional programming may be the production of individuals better prepared to use of some feedstuffs supplying or lacking in specific nutrients, such as VO and plant protein sources. For instance, specific fat and dietary fatty acid supply during embryonic and offspring development may adjust fish metabolism for a better utilisation of 18 carbon fatty acids later in life. Thus, parental nutritional interventions can modulate epigenetic mechanisms that control "metabolic decisions" that are meiostatically and mitotically stable through life in humans (Öst and Pospisilik, 2015), rodents rodents (Morgan *et al.*, 1999; Lillycrop *et al.*, 2005), cattle (Mossa *et al.*, 2015). In fish, nutritional programming studies have mostly focussed on early feeding (Vagner *et al.*, 2007; Vagner *et al.*, 2007; Vagner *et al.*, 2009; Geurden *et al.*, 2013; Rocha *et al.*, 2014; Clarkson *et al.*, 2017; Mellery *et al.*, 2017; Turkmen *et al.*, 2017), whereas parental nutritional interventions are scarcer (Morais *et al.*, 2014; Izquierdo *et al.*, 2015; Otero-Ferrer *et al.*, 2016)

Gilthead sea bream (*Sparus aurata*), is a multi-batch spawner whose oligolecitic eggs largely depend on their continuous intake of nutrients during reproduction (Fernández-Palacios *et al.*, 2011). For this reason, egg nutrient content of gilthead sea bream can be markedly affected by the parental diet even during the spawning season in turn affecting early embryonic development (Izquierdo *et al.*, 2001). Our previous studies have demonstrated that feeding gilthead sea bream broodstock with high linseed oil (LO) diets markedly affect fecundity, spawn quality and growth of 45 days after hatch (dah) larvae and 4-month-old juveniles (Izquierdo *et al.*, 2015). Interestingly, when 4 month-old juveniles were challenged with a low FM and FO diet, offspring from parents fed a 60% FO replacement by LO showed a faster growth and better feed utilisation than those whose parents had been fed with FO (Izquierdo *et al.*, 2015). However, the potential persistence of the effects of broodstock nutritional history on the offspring later in life is still unknown.

Little is known also on the physiological or molecular mechanisms involved in the effect of parental diets on the metabolic performance of the offspring. When n-3 LC-PUFA are limited and the 18C atoms fatty acids are available in gilthead sea bream diet, the gene of fatty acid desaturase 2 (fads2), the key-limiting enzyme for LC-PUFA synthesis, is up-regulated (Izquierdo *et al.*, 2008). Long-chain fatty acid synthesis also involves chain elongation catalysed by elongases (Elovl) with different substrate preferences (Monroig *et al.*, 2011). Among them, Elovl6 is a key lipogenic enzyme which elongates long-chain saturated and monounsaturated fatty acids of 12, 14 and 16 carbon atoms, which has received much attention due its importance in metabolic disorders (Matsuzaka and Shimano, 2009). Besides, LC-PUFA may have a direct effect on the expression of other genes related to lipid or carbohydrate metabolism (Clarke, 2001). Lipoprotein lipase (lpl) facilitates the tissue up-take of circulating fatty acids (Bell and Koppe, 2010) from lipoproteins and its expression in the liver can be regulated by n-3 LC-PUFAs (Raclot *et al.*, 1997). The provision of energy is accomplished by β-oxidation of free fatty acids transported into the mitochondria in the form of fatty acyl-carnitine esters by carnitine acyltransferases, such as carnitine palmitoyltransferases

(Sargent *et al.*, 2002). Replacement of FO with VO changes the fatty acid composition of liver and muscle, affecting the β-oxidation capacity and regulating the expression of cptI and cptII genes (Kjaer *et al.*, 2008; Leaver *et al.*, 2008; Vestergren *et al.*, 2012; Xue *et al.*, 2015). β-oxidation also takes place in the peroxisome and is modulated by peroxisome proliferator activator receptors (PPARs). Three different PPAR isoforms (α, β, γ) have been characterised in gilthead sea bream, ppar being the major form expressed in the liver (Leaver *et al.*, 2008). PPARs are nuclear receptors that regulate differentiation, growth, and metabolism and in mammals, epigenetic mechanisms have been described to regulate these processes involving all the PPARs isoforms (Corbin, 2011). For instance, feeding pregnant rats a protein-restricted diet reduces methylation of the ppar promoter in the offspring and the hypomethylation persists into adulthood (Lillycrop *et al.*, 2008). Finally, another gene potentially regulated by LC-PUFA is cyclooxygenase-2 (cox2), a key enzyme in prostanoid biosynthesis (Ishikawa and Herschman, 2007).

The objective of the present study was to explore the potential persistence of the nutritional programming through parental feeding in offspring later in life and to analyse the physiological or molecular mechanisms implied. For that purpose, the offspring of gilthead sea bream broodstock fed diets with different FO/LO levels, was followed for 18 months until the beginning of first gonad development and nutritionally challenged at 4 and 16 months with low FM and FO diets. The effects of both broodstock feeding and nutritional challenge on growth, chemical and fatty acid composition of muscle and liver, and expression of selected genes in the liver were investigated.

5.2 Materials and methods

5.2.1 Experimental Animals

All fish were obtained from spontaneous spawns of gilthead sea bream broodstock fed 3 diets with 3 levels of FO substitution with LO: 100% FO (F), 40% FO-60% LO (LL), 20%FO-80% LO (HL) (Izquierdo *et al.*, 2015). Offspring from all the groups were fed the same commercial diet during larval rearing, weaning and on growing period until they reached 4 months-old (120 days) (Izquierdo *et al.*, 2015). At this stage, triplicate groups of juveniles were nutritionally challenged for 1 month with either: a high FM/FO diet (f) or a high VM/LO diet (v) named as "reminder diet" in the present study. Details of the broodstock feeding, juvenile nutritional challenge (reminder) at 4 months and feed formulation have been reported earlier (Izquierdo *et al.*, 2015). After this first nutritional challenge (reminder), fish were maintained separately in 1000 L tanks and fed the same commercial diet until they were 16-month-old to be used in the present study. A schematic view of this nutritional programming history is shown in Figure 5-1.

In the present study, 16-month-old offspring seabreams of homogeneous weight were selected and distributed into 18, 500 L light grey-fiberglass cylinder tanks (2.8 kg / m^3). Each tank contained 30 fish with an initial body weight of 243.2 ± 12.7 g (mean±SD). Tanks were supplied with filtered seawater (37 ppm salinity), which entered from the tank surface and drained from the bottom at a rate of 250 L/h to maintain high water quality, which was tested daily, and no deterioration was observed. O_2 level, water temperature and pH were monitored real time by Miranda aquaculture water quality monitoring system (Innovaqua, Sevilla, Spain). Water was continuously aerated (125 mL/min), attaining an average of 6.8±0.8 ppm dissolved O_2 during the experimental period. Average water temperature and pH along the trial were 24.6±0.6°C and 7.89, respectively. Natural photoperiod was kept during the whole experimental period (10 h light).

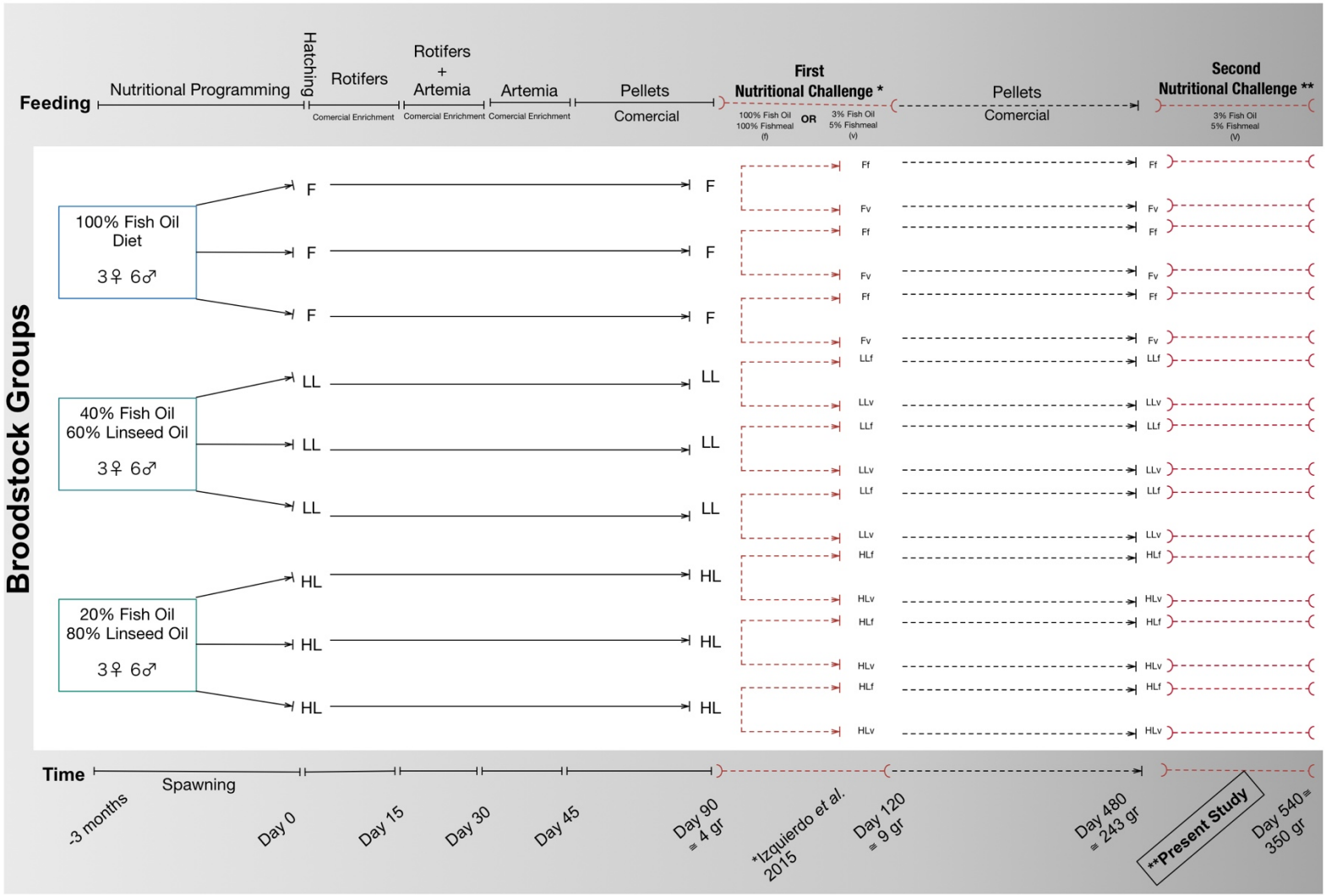

Figure 5-1 | Schematic view of nutritional programming history of gilthead sea bream

5.2.2 Experimental Diet

The experimental diet was formulated and produced by Biomar (Denmark) to be low in FO (3%) and FM (5%). Thus, the diet was high in oleic (18:1n-9), LA (18:2n-6), and ALA (18:3n-3) acids (Table 5-1). Juveniles from each group were daily fed until apparent satiation for 60 days, three times a day at 09:00, 13:00 and 17:00 hours.

Table 5-1 I Main ingredients*, energy, protein and % total fatty acids contents of diet for the nutritional challenge of gilthead seabream juveniles obtained from broodstock fed diets 100% FO, 40%FO-60%LO and 20%FO-80%LO (F, LL and HL) during spawning

Main Ingredients (%)	%	Proximate Composition	(% dry matter)
Fish meal SA[1] 68 super prime	5.00	Crude lipids	21.7
Fish meal alternative protein sources[2]	54.50	Crude protein	45.1
Rapeseed meal cake	11.30	Moisture	9.0
Wheat	6.89	Ash	5.4
Fish oil SA[1]	3.00		
Vegetable oil mix[3]	13.00	Gross Energy (MJ/kg, as is)	22.5
% total fatty acids		% total fatty acids	
14:0	6.6	18:3n-3	11.8
14:1n-5	0.1	18:4n-3	0.4
15:0	0.1	18:4n-1	0.0
16:0ISO	0.0	20:0	0.4
16:0	12.3	20:1n-9	0.0
16:1n-7	2.1	20:1n-7	1.0
16:1n-5	0.1	20:1n-5	0.1
16:2n-4	0.2	20:2n-9	0.0
17:0	0.3	20:2n-6	0.1
16:3n-4	0.1	20:3n-9	0.0
16:3n-3	0.0	20:3n-6	0.0
16:3n-1	0.0	20:4n-6	0.2
16:4n-3	0.4	20:3n-3	0.0
18:0	3.2	20:4n-3	0.1
18:1n-9	32.3	20:5n-3	2.5
18:1n-7	2.3	22:1n-11	0.1
18:1n-5	0.0	22:1n-9	0.3
18:2n-9	0.0	22:4n-6	0.0
18:2n-6	20.3	22:5n-6	0.1
18:2n-4	0.1	22:5n-3	0.3
18:3n-6	0.1	22:6n-3	1.7
18:3n-4	0.0		

* Please see Torrecillas *et al.*, (for the complete list of feed ingredients.
[1] South American, Superprime (Feed Service, Bremen, Germany).
[2] Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
[3] Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

The feed was supplied in small portions (< 5-6 pellets at a time) to ensure that all feed was eaten. After each feeding, uneaten feed was collected, kept in aluminium oven trays, dried overnight at 105 ºC and weighted to calculate feed intake.

5.2.3 Biochemical Analyses

Moisture, protein (AOAC, 1995) and crude lipid (Folch *et al.*, 1957) contents of the tissue samples and diets were analysed. Fatty acid methyl esters were obtained by trans methylation of crude lipids as previously described (Christie, 1982). Fatty acid methyl esters were separated by gas-liquid chromatography (GC -14A, Shimadzu, Tokyo, Japan) following the conditions described previously (Izquierdo *et al.*, 1990) and identified by comparison to previously characterised standards and GLC-MS (Polaris QTRACE™ Ultra, Thermo Fisher Scientific, MA, USA).

5.2.4 Molecular Studies

Liver samples from 3 fish per each tank (9 per group) were collected at the beginning (480 days-old fish) and at the end of the feeding challenge (540 days-old fish). Samples were collected on ice from fish kept unfed for 24 h, each tissue sample from one individual assigned to corresponding 1.5 mL Eppendorf tube and were snap frozen in liquid nitrogen immediately after sampling of each individual fish. The samples were then conserved at -80°C until RNA extraction and analyses. RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). Prior to real-time PCR analysis two different potential housekeeping genes, beta-actin (*ß-act)* and ribosomal protein L27 *(rpl27*), were tested. Data from duplicate samples (n=18) using the two candidate housekeeping genes were compared by an online program (http://leonxie.esy.es/RefFinder/?type=reference) (RefFinder) (Xie *et al.*, 2012) and *ß-act* was selected as the most suitable housekeeping gene for the present study (*ß-act*, threshold cycle (Ct) values min=18.9, max=20.6, mean=19.64, SD=0.5 - *rpl27*, min=17.0 max=21.2, mean=19.1, SD=1.01).

Real-time quantitative PCRs were performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using *ß-act* as the housekeeping gene

in a final volume of 15 µL per reaction well, and 100 ng of total RNA reverse transcribed to complementary cDNA. Samples, housekeeping gene, cDNA template and reaction blanks were analysed in duplicates (Table 2).

Table 5-2 | Primers, RT-PCR reaction efficiencies, and GeneBank accession numbers and reference articles for sequences of target and housekeeping genes

Gene*	Primer Sequence 5'-3' (F) and 5'-3' (R)	Efficiency (%)	GenBank Access No.	Reference
lpl	CGT TGC CAA GTT TGT GAC CTG AGG GTG TTC TGG TTG TCT GC	98.0	AY495672	(Benedito-Palos *et al.*, 2014)
ppara	TCT CTT CAG CCC ACC ATC CC ATC CCA GCG TGT CGT CTC C	102.0	AY590299	(Benedito-Palos *et al.*, 2014)
elovl6	GTG CTG CTC TAC TCC TGG TA ACG GCA TGG ACC AAG TAG T		JX975702	(Benedito-Palos *et al.*, 2014)
fads2	CGA GAG CCA CAG CAG CAG GGA CGG CCT GCG CCT GAG CAG TT	109.2	AY055749	(Turkmen *et al.*, 2017)
cox2	GAG TAC TGG AAG CCG AGC AC GAT ATC ACT GCC GCC TGA GT	107.0	AM296029	(Sepulcre *et al.*, 2007)
cpt1b	CCA CCA GCC AGA CTC CAC AG CAC CAC CAG CAC CCA CAT ATT TAG	98.0	DQ866821	(Boukouvala *et al.*, 2010)
ß-act	TCT GTC TGG ATC GGA GGC TC AAG CAT TTG CGG TGG ACG	100.7**	X89920	-

*Complete gene names; *lpl*: lipoprotein lipase, *ppara*: peroxisome proliferator-activated receptor alpha, *elovl6*: elongation of very long chain fatty acids protein 6, *fads2*: fatty acid desaturase 2, *cox2*: cyclooxygenase-2, *cpt1*: carnitina palmitoiltransferase I, *ß-act*: beta-actin.
**The average efficiency of housekeeping gene from 6 RT-PCR runs.

Primer efficiency was tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:100 and 1:1000). One 96 well PCR plate used to analyse each gene, primer efficiency and blank samples (Bio-rad, Multiplate, CA, USA). Melting curve was performed and amplification of single product was confirmed after each run. Fold expression of each gene was determined by delta–delta C_T method ($2^{-\Delta\Delta CT}$) (Izquierdo *et al.*, 1990). PCR efficiencies were similar and no efficiency correction was required (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) (Table 2). Fold expression was related to that of offspring obtained from FO diet fed broodstock and fed commercial diets through their life (Ff group).

5.2.5 Statistical Analysis

Data on growth and biochemical composition were statistically analysed by two-way ANOVA, using broodstock diet and reminder diet as fixed factors in IBM SPSS

v23.0.0.2 for Mac (IBM SPSS Inc. Chicago, IL, USA). Data were split into groups based on each fixed factor (broodstock and reminder diet) and compared with one-way ANOVA. Scheffe post hoc multiple comparisons for broodstock and reminder diet, separately, assessed differences between groups. Prior to analysis of the data, equality of variances was tested by Levene's test and distribution of data by Shapiro-Wilk tests. All data except *cox2* gene expression showed normal distribution and equality of variances.

Gene expression data (except for *cox2*) were analysed by two-way ANOVA using broodstock and reminder diet as fixed factors. Expression data then were analysed by Welch's ANOVA then compared with Games-Howell test for identification of differences between groups. Fixed factor for Welch's ANOVA was experimental groups in gene expression data analysis. Pearson correlation test was performed using R (R Foundation for Statistical Computing, Vienna, Austria). Gene expression figures were created using lattice package (version 0.20-33) (Sarkar, 2008) downloaded from the comprehensive R Archive Network (CRAN) library. Sample size for all analysed data was 9 and data were expressed as mean ± standard deviation (SD).

5.3 Results

5.3.1 Growth Performance

The low FM/FO diet was well accepted and there were no significant differences in feed intake among fish groups (3.95 ± 0.39 kg, mean±SD $P>0.05$). At the beginning of the trial, there were no significant differences in fish body weight (243.2 ± 12.7 g, mean±SD) among experimental groups ($P>0.05$) (Table 5-3). However, after 60 days of feeding the low FM/FO diet, LLv fish (obtained from broodstock fed low LO and fed at 4 months with the reminder diet v, high in VM and VO) showed the highest body weight, being significantly ($P<0.05$) higher than that of fish LLf, from the same broodstock but fed at 4 months the diet f (high in FM and FO) (Table 5-3). Besides, growth of LLv fish was also significantly higher than that of fish from Fv or HLv, which had been fed the same reminder diet v, but came from broodstock fed FO or high LO. Thus, the two-way

ANOVA analysis of final body weight showed a significant effect of the broodstock diet ($P<0.05$) and the interaction between broodstock and reminder diet ($P<0.01$). The specific growth rate of LLv fish was significantly higher than that of LLf, denoting the significant effect of the reminder diet (diet v), as well as higher than Fv and HLv ($P<0.05$).

Table 5-3 l Growth performance parameters after 2 months feeding of very low fish meal (5%) and fish oil (3%) diet in 16 month-old gilthead seabream (Sparus aurata) originated from broodstock fed linseed oil in replacement of fish oil: 0% (F), 60% (LL), 80% (HL) (mean±SD, n=3) and fed either a fish meal and fish oil based diet (f) or a very low fish meal (5%) and fish oil (3%) "reminder" diet (v) for 1 month at 4 months of age

Groups	Initial Body Weight (g)	Final Body Weight (g)	SGR[1] (%/day)	FCR[2]
Ff	243.0±1.9	337.4±3.3	0.7±0.06	^{A}1.5±0.1
LLf	244.1±0.9	^{B}336.3±2.6	^{B}0.6±0.03	^{B}1.7±0.1
HLf	242.5±2.0	339.6±7.2	0.7±0.05	1.6±0.1
Fv	243.6±0.9	319.5±5.5^{b}	0.5±0.07^{b}	^{B}1.8±0.1^{b}
LLv	246.4±0.4	^{A}351.3±1.2^{a}	^{A}0.7±0.03^{a}	^{A}1.4±0.2^{a}
HLv	242.3±1.3	321.3±4.0^{b}	0.6±0.07^{b}	1.9±0.2^{b}
*Two-way ANOVA**				
Broodstock Diet (B)	*n.d.*	*p<0.05*	*n.d.*	*n.d.*
Reminder Diet (R)	*n.d.*	*n.d.*	*p<0.05*	*n.d.*
B × R	*n.d.*	*p<0.01*	*P<0.05*	*p<0.01*

* Capital superscript letters indicate differences between fish fed f or v diets during the first nutritional challenge (reminder) coming from the same parental feeding. Low case superscript letters indicate differences between fish coming from different parental feeding and fed the same diet during the first nutritional challenge (reminder) ($P<0.05$). All values are mean ± SD (n=3). n.d. no difference.
[1]Specific growth rate (**SGR** %/day) = [Ln (final weight. g) – Ln (initial weight. g)] / (number of days) × 100.
[2]Feed conversion ratio (**FCR**) = (total weight of consumed feed. g) / (weight gain. g).

Thus, the two-way ANOVA showed the significant effect of the reminder diet at 4 months-old as well as the interaction between broodstock and reminder diet ($P<0.05$). Regarding feed conversion, the best values were also obtained in fish LLv, Fv or HLv. For fish coming from broodstock fed FO, the feed conversion ratio (FCR) was better when fish had been fed FO at 4-months (Ff) than those fed v diet (Fv). The two-way ANOVA showed a strong interaction between broodstock and reminder diets ($P<0.01$).

5.3.2 Biochemical Composition

At the end of the study, protein, lipid and ash contents of liver or muscle were similar (P>0.05) (Table 5-4).

Table 5-4 | Biochemical composition of liver and muscle tissue after 2 months feeding a very low fish meal (5%) and fish oil (3%) diet in 16 month-old gilthead seabream (Sparus aurata) originated from broodstock fed linseed oil in replacement of fish oil: 0% (F), 60% (LL), 80% (HL) (mean±SD, n=3) and fed either a fish meal and fish oil based diet (f) or a very low fish meal (5%) and fish oil (3%) "reminder" diet (v) for 1 month at 4 months of age

	Groups	Moisture (%)	Protein (%)	Lipids (%)	Ash (%)
LIVER*	Ff	64.2±2.0	11.1±0.3	14.9±3.0	2.5±0.5
	LLf	66.6±5.9	10.1±2.4	12.9±5.9	2.7±1.2
	HLf	66.5±0.1	12.1±0.4	11.7±1.8	2.9±0.3
	Fv	66.6±4.2	11.2±1.3	11.0±2.2	2.9±0.4
	LLv	68.4±2.8	12.2±1.4	10.9±4.9	2.7±0.8
	HLv	67.1±4.1	10.7±1.3	12.7±4.4	2.4±0.1
MUSCLE*	Ff	72.3±0.1	21.6±0.1	5.1±0.5	1.4±0.1
	LLf	72.7±0.5	20.6±0.9	5.2±0.7	1.4±0.1
	HLf	72.5±0.8	21.3±0.2	5.4±1.1	1.4±0.1
	Fv	73.0±0.1	21.3±0.3	4.7±0.2	1.5±0.1
	LLv	72.5±1.0	21.3±0.4	5.2±0.5	1.5±0.1
	HLv	72.9±0.1	21.4±0.2	4.9±0.5	1.4±0.1

*All values are mean ± SD (n=3). No significant differences were found for broodstock diet (P>0.05), reminder diet (P>0.05) and interaction of these two factors (P>0.05) by the two-way ANOVA analysis.

However, liver fatty acid composition was significantly affected by broodstock or reminder diets as well as by their interaction (Table 5-5). For instance, LO increase in broodstock diet significantly reduced liver contents on 16:4n-3, a product of EPA ß-oxidation, and increased 16:3n-1 or 18:0, whereas the interaction of broodstock and reminder diets affected the ratios 18:0/16:0 and 18:1/16:1, indicators of *elovl6* activity, and the related ratio 16:1/16:0 (Table 5-5). The *fads2* products 20:3n-6 and 20:4n-3 were significantly reduced by the reminder diet (P=0.018) and its interaction with the broodstock diet (P=0.015), respectively, whereas 20:4n-6, 20:5n-3 and 22:6n-3 tend to

be higher in offspring fed the reminder diet, but were not significantly different (P=0.17) (Table 5-5). Muscle fatty acid composition did not differ significantly among the different experimental groups (P>0.05) (Table 5-6).

Table 5-5 l % total fatty acids of livers after 2 months feeding a very low fish meal (5%) and fish oil (3%) diet in 16 month-old gilthead seabream (Sparus aurata) originated from broodstock fed linseed oil in replacement of fish oil: 0% (F), 60% (LL), 80% (HL) (mean±SD, n=3) and fed either a fish meal and fish oil based diet (f) or a very low fish meal (5%) and fish oil (3%) "reminder" diet (v) for 1 month at 4 months of age

	Groups						Two-way ANOVA*		
	Ff	LLf	HLf	Fv	LLv	HLv	B	R	B×R
FA (%)									
14:0	4.17±0.53	4.68±0.33	5.29±0.72	4.86±0.59	4.89±0.11	4.68±0.90	*0.403*	*0.732*	*0.201*
14:1n-7	0.05±0.01	0.05±0.00	0.05±0.00	0.05±0.01	0.05±0.00	0.04±0.00	*0.745*	*0.477*	*0.480*
14:1n-5	0.06±0.00	0.07±0.02	0.05±0.01	0.07±0.02	0.06±0.01	0.06±0.01	*0.581*	*0.485*	*0.206*
15:0	0.16±0.01	0.20±0.05	0.17±0.03	0.18±0.03	0.18±0.02	0.19±0.00	*0.506*	*0.409*	*0.333*
15:1n-5	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	*0.625*	*0.220*	*0.654*
16:0ISO	0.03±0.00	0.04±0.01	0.03±0.00	0.03±0.00	0.03±0.00	0.04±0.01	*0.888*	*0.851*	*0.241*
16:0	12.61±0.93	14.33±1.96	14.81±0.83	13.92±0.71	14.77±0.30	13.63±0.64	*0.141*	*0.717*	*0.200*
16:1n-7	4.51±0.15	4.89±0.84	4.00±0.07	4.76±0.54	4.37±0.10	4.70±0.69	*0.557*	*0.545*	*0.148*
16:1n-5	0.10±0.01	0.11±0.01	0.09±0.00	0.10±0.01	0.10±0.00	0.11±0.02	*0.476*	*0.401*	*0.038*
16:2n-6	0.00±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	*0.230*	*0.735*	*0.040*
16:2n-4	0.21±0.00	0.24±0.05	0.17±0.02	0.23±0.05	0.21±0.02	0.23±0.00	*0.542*	*0.329*	*0.071*
17:0	0.19±0.00	0.20±0.01	0.15±0.01	0.20±0.04	0.18±0.01	0.20±0.01	*0.259*	*0.165*	*0.112*
16:3n-4	0.18±0.01	0.18±0.01	0.16±0.00	0.17±0.00	0.16±0.00	0.17±0.03	*0.158*	*0.208*	*0.058*
16:3n-3	0.05±0.00	0.06±0.02	0.05±0.00	0.05±0.00	0.05±0.00	0.06±0.01	*0.805*	*0.655*	*0.287*
16:3n-1	0.01±0.00	0.02±0.00	0.02±0.00	0.01±0.00	0.02±0.00	0.01±0.00	*0.015*	*0.787*	*0.600*
16:4n-3	0.12±0.01	0.11±0.01	0.07±0.01	0.12±0.04	0.11±0.01	0.09±0.00	*0.037*	*0.609*	*0.437*
16:4n-1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	*0.441*	*0.377*	*0.441*
18:0	4.28±0.26	4.15±0.30	4.97±0.22	3.88±0.37	4.5±0.32	4.41±0.42	*0.026*	*0.206*	*0.068*
18:1n-9	31.93±1.02	29.93±2.77	32.06±0.48	30.14±3.42	29.6±2.52	30.1±2.38	*0.571*	*0.259*	*0.812*
18:1n-7	2.72±0.06	2.85±0.18	2.66±0.06	2.68±0.15	2.64±0.07	2.88±0.26	*0.671*	*0.921*	*0.065*
18:1n-5	0.12±0.01	0.13±0.03	0.09±0.01	0.12±0.03	0.10±0.00	0.13±0.02	*0.762*	*0.911*	*0.045*
18:2n-9	0.61±0.13	0.57±0.28	0.44±0.02	0.62±0.07	0.53±0.19	0.55±0.27	*0.546*	*0.791*	*0.793*
18:2n-6	13.1±0.47	12.92±1.26	13.87±0.81	12.93±1.02	12.86±0.18	12.93±0.95	*0.617*	*0.375*	*0.672*
18:2n-4	0.15±0.01	0.16±0.01	0.13±0.00	0.14±0.02	0.14±0.01	0.16±0.02	*0.943*	*0.653*	*0.047*
18:3n-6	1.08±0.21	1.04±0.45	0.78±0.06	1.08±0.08	0.91±0.21	0.93±0.31	*0.375*	*0.944*	*0.665*
18:3n-4	0.15±0.00	0.15±0.01	0.12±0.00	0.15±0.02	0.13±0.01	0.14±0.02	*0.063*	*0.743*	*0.216*
18:3n-3	5.32±0.36	5.03±1.19	5.74±0.28	5.15±0.78	5.31±0.06	5.03±1.16	*0.891*	*0.577*	*0.556*
18:3n-1	0.01±0.00	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00	*0.392*	*0.491*	*0.173*
18:4n-3	0.72±0.07	0.75±0.25	0.55±0.03	0.77±0.13	0.66±0.08	0.66±0.02	*0.222*	*0.765*	*0.444*
18:4n-1	0.14±0.01	0.14±0.01	0.11±0.01	0.12±0.02	0.12±0.00	0.14±0.00	*0.323*	*0.671*	*0.039*
20:0	0.12±0.01	0.13±0.03	0.13±0.02	0.12±0.01	0.14±0.01	0.14±0.02	*0.308*	*0.764*	*0.970*
20:1n-9	0.16±0.02	0.18±0.06	0.11±0.02	0.18±0.07	0.15±0.02	0.19±0.05	*0.720*	*0.352*	*0.141*
20:1n-7	1.37±0.11	1.41±0.42	1.28±0.07	1.28±0.01	1.33±0.12	1.40±0.04	*0.903*	*0.869*	*0.629*
20:1n-5	0.13±0.01	0.14±0.03	0.11±0.01	0.12±0.01	0.12±0.01	0.14±0.02	*0.803*	*0.835*	*0.232*
20:2n-9	0.78±0.07	0.53±0.09	0.57±0.08	0.55±0.18	0.55±0.06	0.48±0.10	*0.098*	*0.068*	*0.16*
20:2n-6	0.59±0.08	0.54±0.04	0.63±0.03	0.49±0.10	0.55±0.06	0.56±0.06	*0.385*	*0.156*	*0.314*
20:3n-9	0.02±0.02	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.00	0.02±0.01	*0.709*	*0.533*	*0.409*
20:3n-6	0.52±0.08	0.39±0.06	0.37±0.04	0.34±0.07	0.35±0.02	0.37±0.04	*0.144*	*0.018*	*0.048*
20:4n-6	0.41±0.06	0.50±0.15	0.42±0.05	0.47±0.15	0.57±0.18	0.49±0.07	*0.450*	*0.312*	*0.998*
20:3n-3	0.45±0.05	0.39±0.03	0.47±0.03	0.37±0.09	0.42±0.04	0.43±0.09	*0.448*	*0.277*	*0.308*
20:4n-3	0.72±0.08	0.64±0.06	0.51±0.04	0.59±0.09	0.56±0.05	0.64±0.06	*0.136*	*0.421*	*0.015*
20:5n-3	2.85±0.25	3.01±0.28	2.16±0.23	3.09±1.22	3.11±0.48	3.10±0.02	*0.489*	*0.168*	*0.491*
22:1n-11	0.48±0.10	0.61±0.33	0.33±0.09	0.52±0.11	0.52±0.10	0.57±0.01	*0.525*	*0.479*	*0.301*
22:1n-9	0.40±0.01	0.43±0.10	0.40±0.04	0.38±0.00	0.41±0.03	0.43±0.01	*0.576*	*0.943*	*0.618*
22:4n-6	0.08±0.01	0.08±0.02	0.06±0.00	0.07±0.02	0.07±0.01	0.09±0.01	*0.875*	*0.301*	*0.118*
22:5n-6	0.07±0.01	0.08±0.01	0.06±0.01	0.07±0.03	0.08±0.01	0.08±0.00	*0.587*	*0.217*	*0.514*
22:5n-3	2.25±0.47	1.98±0.21	1.36±0.24	2.06±0.91	1.79±0.21	2.25±0.28	*0.457*	*0.487*	*0.152*
22:6n-3	5.78±1.09	5.90±1.15	4.35±0.66	6.67±3.09	6.55±1.37	6.33±0.35	*0.612*	*0.168*	*0.785*
∑ SAT·	21.55±1.23	23.73±2.49	25.55±0.81	23.21±1.48	24.7±0.62	23.28±1.97	*0.088*	*0.876*	*0.135*
∑ MUFA·	42.06±0.91	40.83±0.77	41.26±0.71	40.44±2.58	39.47±2.28	40.78±3.51	*0.592*	*0.243*	*0.882*
∑ n-6·	15.84±0.32	15.55±1.54	16.19±0.77	15.47±0.92	15.39±0.19	15.45±0.71	*0.806*	*0.343*	*0.859*
∑ n-3·	18.28±1.56	17.91±0.39	15.27±1.25	18.88±4.61	18.58±1.98	18.6±1.25	*0.510*	*0.209*	*0.582*
18:0/16:0	0.34±0.04	0.29±0.02	0.34±0.02	0.28±0.02	0.3±0.02	0.32±0.02	*0.204*	*0.089*	*0.064*
18:1/16:1	1.18±0.06	1.2±0.11	1.02±0.13	1.19±0.22	0.94±0.05	0.92±0.02	*0.275*	*0.633*	*0.047*

Values in the graphs are presented as mean and standard deviation (n=3).
*B = Broodstock, R= Reminder, B×R= Interaction of broodstock & reminder, P values under 0.05 are bolded.
∑ Saturated fatty acids include 14:0, 15:0, 16:0, 17:0, 18:0 & 20:0.
·∑ MUFA: Mono unsaturated fatty acids include 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11 & 22:1n-9.
·∑ n-6: n-6 series polyunsaturated fatty acids include 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6.
·∑ n-3: n-3 series polyunsaturated fatty acids include 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3.

Table 5-6 l % total fatty acids of muscle after 2 months feeding a very low fish meal (5%) and fish oil (3%) diet in 16 month-old gilthead seabream (Sparus aurata) originated from broodstock fed linseed oil in replacement of fish oil: 0% (F), 60% (LL), 80% (HL) (mean±SD, n=3) and fed either a fish meal and fish oil based diet (f) or a very low fish meal (5%) and fish oil (3%) "reminder" diet (v) for 1 month at 4 months of age

	Groups					
	Ff	LLf	HLf	Fv	LLv	HLv
FA (%)*						
14:0	4.83±0.15	4.90±0.15	5.17±0.15	4.99±0.33	4.98±0.03	4.97±0.35
14:1n-7	0.08±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.07±0.00	0.08±0.00
14:1n-5	0.08±0.00	0.09±0.03	0.08±0.02	0.09±0.01	0.08±0.00	0.08±0.01
15:0	0.21±0.01	0.23±0.01	0.21±0.01	0.22±0.02	0.22±0.00	0.22±0.02
15:1n-5	0.02±0.00	0.03±0.00	0.03±0.00	0.02±0.01	0.03±0.00	0.03±0.00
16:0ISO	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.01
16:0	14.69±0.42	15.03±0.41	14.52±0.21	15.03±0.12	15.18±0.31	14.88±0.48
16:1n-7	4.89±0.31	5.12±0.18	4.51±0.14	4.92±0.64	4.89±0.36	4.87±0.72
16:1n-5	0.08±0.00	0.09±0.00	0.08±0.00	0.08±0.01	0.08±0.01	0.08±0.01
16:2n-6	0.05±0.04	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
16:2n-4	0.36±0.07	0.33±0.02	0.29±0.01	0.31±0.05	0.31±0.01	0.31±0.04
17:0	0.28±0.03	0.30±0.01	0.27±0.00	0.29±0.04	0.28±0.01	0.29±0.03
16:3n-4	0.14±0.00	0.14±0.00	0.14±0.01	0.14±0.01	0.14±0.00	0.14±0.01
16:3n-3	0.05±0.00	0.05±0.01	0.04±0.00	0.05±0.02	0.04±0.01	0.05±0.01
16:3n-1	0.05±0.02	0.07±0.01	0.07±0.01	0.09±0.01	0.07±0.00	0.08±0.02
16:4n-3	0.35±0.11	0.34±0.03	0.31±0.01	0.33±0.06	0.34±0.01	0.30±0.09
16:4n-1	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.01
18:0	3.50±0.04	3.45±0.11	3.55±0.11	3.55±0.07	3.53±0.00	3.61±0.10
18:1n-9	27.27±0.21	27.23±0.92	28.47±0.99	27.23±1.37	27.77±0.28	27.66±1.00
18:1n-7	2.85±0.06	2.90±0.01	2.74±0.04	2.84±0.16	2.82±0.08	2.84±0.14
18:1n-5	0.39±0.45	0.13±0.01	0.11±0.01	0.13±0.02	0.12±0.01	0.13±0.02
18:2n-9	0.22±0.02	0.25±0.06	0.22±0.01	0.24±0.02	0.24±0.02	0.22±0.02
18:2n-6	11.52±0.40	11.46±0.55	12.77±0.49	11.62±1.36	11.74±0.62	11.79±1.59
18:2n-4	0.13±0.01	0.14±0.00	0.13±0.01	0.13±0.01	0.13±0.01	0.14±0.01
18:3n-6	0.35±0.05	0.36±0.05	0.33±0.01	0.35±0.03	0.34±0.02	0.32±0.00
18:3n-4	0.15±0.01	0.14±0.00	0.13±0.00	0.15±0.01	0.13±0.01	0.14±0.01
18:3n-3	5.11±1.11	4.53±0.51	5.48±0.47	4.57±1.16	4.76±0.40	4.75±1.18
18:3n-1	0.18±0.30	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
18:4n-3	0.69±0.04	0.73±0.03	0.64±0.03	0.69±0.07	0.70±0.05	0.67±0.06
18:4n-1	0.22±0.13	0.14±0.00	0.14±0.01	0.13±0.01	0.14±0.01	0.15±0.01
20:0	0.24±0.01	0.24±0.01	0.24±0.01	0.24±0.00	0.24±0.01	0.24±0.00
20:1n-9	0.28±0.02	0.29±0.03	0.25±0.03	0.31±0.09	0.28±0.03	0.28±0.07
20:1n-7	2.36±0.13	2.44±0.24	2.19±0.10	2.43±0.38	2.37±0.11	2.35±0.34
20:1n-5	0.16±0.01	0.17±0.01	0.16±0.01	0.17±0.02	0.16±0.01	0.16±0.02
20:2n-9	0.31±0.01	0.31±0.02	0.31±0.03	0.30±0.02	0.30±0.01	0.28±0.01
20:2n-6	0.60±0.29	0.42±0.02	0.45±0.00	0.42±0.02	0.42±0.00	0.43±0.02
20:3n-9	0.03±0.01	0.03±0.01	0.05±0.01	0.04±0.02	0.04±0.03	0.06±0.03
20:3n-6	0.23±0.00	0.22±0.01	0.24±0.02	0.22±0.01	0.21±0.01	0.21±0.03
20:4n-6	0.46±0.04	0.46±0.04	0.42±0.06	0.46±0.06	0.44±0.00	0.46±0.05
20:3n-3	0.33±0.08	0.28±0.00	0.32±0.00	0.28±0.04	0.28±0.01	0.30±0.04
20:4n-3	0.58±0.00	0.58±0.01	0.54±0.02	0.55±0.05	0.55±0.03	0.57±0.05
20:5n-3	4.36±0.21	4.42±0.19	3.88±0.27	4.15±0.62	4.22±0.17	4.26±0.61
22:1n-11	1.42±0.16	1.53±0.24	1.21±0.12	1.52±0.40	1.45±0.15	1.41±0.36
22:1n-9	0.56±0.02	0.57±0.06	0.53±0.01	0.59±0.07	0.56±0.01	0.55±0.05
22:4n-6	0.10±0.01	0.10±0.01	0.10±0.01	0.11±0.01	0.10±0.00	0.11±0.01
22:5n-6	0.12±0.01	0.13±0.00	0.11±0.01	0.13±0.01	0.12±0.01	0.13±0.00
22:5n-3	2.07±0.15	2.10±0.20	1.88±0.15	2.08±0.27	1.99±0.10	2.09±0.26
22:6n-3	6.99±0.73	7.41±0.66	6.53±0.89	7.64±1.13	7.03±0.12	7.27±0.81

*All values are mean ± SD (n=3). No significant differences were found for broodstock diet (P>0.05), reminder diet (P>0.05) and interaction of these two factors (P>0.05) by the two-way ANOVA analysis.

5.3.3 Gene Expression

Reduction of LC-PUFA and increase in ALA and LA in broodstock diets lead to a significant (P<0.001) down-regulation of hepatic *lpl* (Figure 5-2), which was significantly (P<0.01) emphasised by feeding the 4 month-old juveniles the reminder v diet, based on plant ingredients and with low LC-PUFA and high ALA and LA contents. Thus, the lowest *lpl* relative expression was found in HLv and HLf fish. Similarly, the origin of the fish by different broodstock diets significantly (P<0.01) down-regulated hepatic *elovl6* (Figure 5-2), with the lowest *elovl6* relative expression found in LLv and HLf fish. Besides, *elovl6* expression was significantly correlated to liver contents of 18:1/16:1 (r=0.89) and 18:0/16:0 (r=0.89), ratios of product/substrate of *elovl6* activity. There was not a significant effect of either the broodstock or the reminder diet on hepatic expression of *fads2* (Figure 2), but their values were positively correlated to hepatic levels of 18:4n-3 (r=0.86) and 18:3n-6 (r=0.8), products of the *fads2* activity, as well as the end desaturation products 20:5n-3 (r=0.98), 22:6n-3 (r=0.95) and 22:5n-6 (r=0.95). Besides, *fads2* expression values were negatively correlated (r=-0.52) to *elovl6.* Regarding fatty acid catabolism biomarkers, reduction of LC-PUFA and increase in ALA and LA acids in broodstock diets lead to a significant (P<0.001) down-regulation of hepatic *cpt1b* (Figure 5-2), which was significantly (P<0.05) emphasised by the reminder diet. Moreover, relative expression of *cpt1b* was highly correlated to 18:1n-9 (r=0.82) and negatively correlated to 20:5n-3 (r=-0.62). No significant differences were found in *ppara* or *cox2* relative expression, which were negatively correlated (r=-0.57 and r=-0.73, respectively) to *cpt1b* expression.

The overall response showed similar trends for *lpl*, *cpt1b* and *elovl6* expressions, whose values showed high correlation in their relative gene expression between *lpl* and *elovl6* (r=0.52), *cpt1b* and *elovl6* (r=0.72) and *lpl* and *cpt1b* (r=0.74).

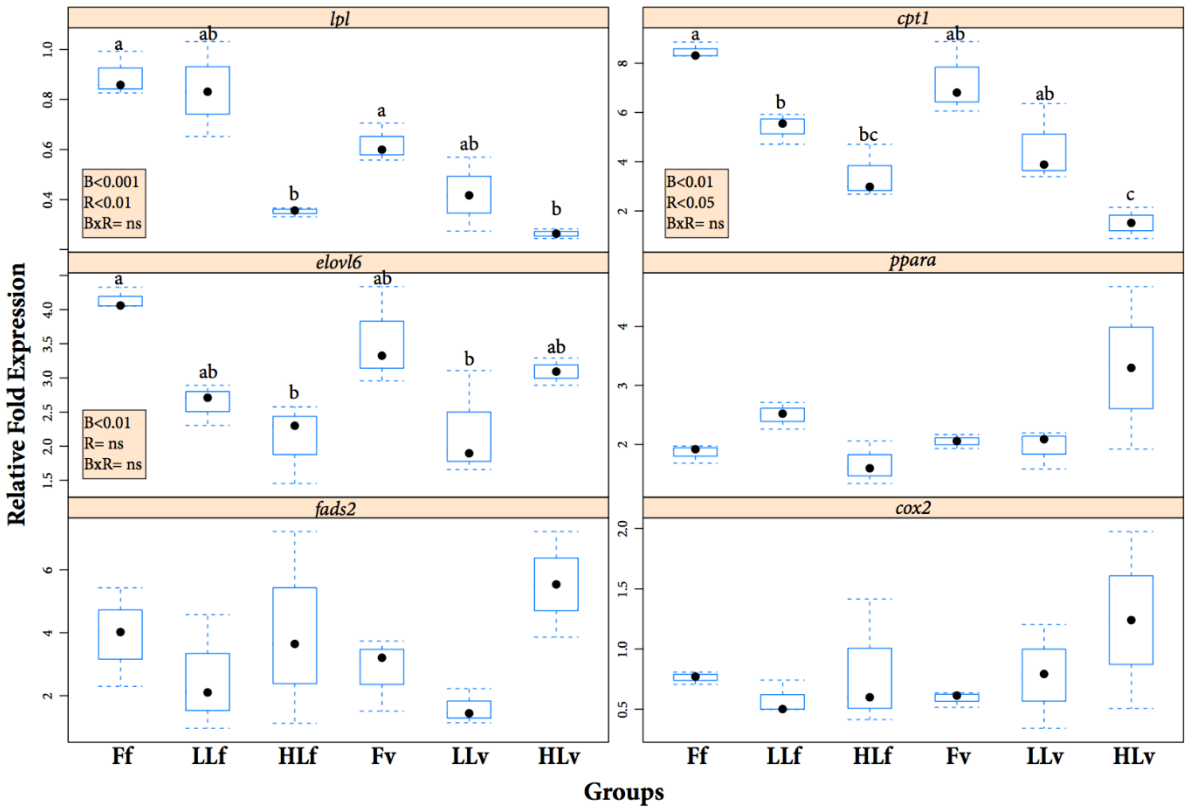

Figure 5-2 I Box-and-whisker plots of relative fold expression (groups vs control sample) of six different genes after challenging 16 month-old gilthead seabream individuals with high vegetable oil and meal feeds for 2 months.

Complete gene names; *lpl*: Lipoprotein lipase, *ppara*: Peroxisome proliferator-activated receptor alpha, *elovl6*: Elongation of very long chain fatty acids protein 6, *fads2*: Fatty acid desaturase 2, *cox2*: Cyclooxygenase-2, *cpt1*: Carnitine palmitoiltransferase I.

Indications are as follows: Dashed lines = maximum and minimum fold expression, boxes = upper and lower quartiles, and dots = median. Dashed box indicates p values of two-way ANOVA, B = Broodstock diet, R = Reminder diet, B x R= interaction of these two parameters, ns: P>0.05. n=3 for all the groups and genes. Letters indicate differences between each group, no indications mean no significance difference (P-value > 0.05).

5.4 Discussion

In animal production, nutritional programming can be useful to improve offspring adaptation to farm conditions (Mathers, 2007; Monaghan, 2008). Since the limited availability of FM and FO is a main constraint in fish production, modulation of offspring phenotype through parental feeding for an improved utilisation of low FM and FO diets can have important advantages (Gotoh, 2015). Previous studies in gilthead sea bream have demonstrated that it is possible to improve low FM and FO feed utilisation in the offspring coming from broodstock fed with increased substitution of FO with LO

(Izquierdo *et al.*, 2015). This adaptation included the regulation of expression of genes for key metabolic enzymes in the liver such as *fads2* (Izquierdo *et al.*, 2015) or glucocorticoid receptor (*gr*) (S. Turkmen *et al.*, in prep.). However, the persistence of these phenotypic or metabolic changes later in life had not been yet studied. The present study shows that parental feeding with moderate FO replacement with LO combined with juvenile feeding with low FM and FO diets improves offspring growth and feed utilisation of low FM/FO diets even when they are 16 months-old, in the verge of their first reproductive season. Thus, among fish fed the low FM/FO diet during the juvenile stages, those obtained from parents fed moderate LO levels showed the highest growth, denoting the persistent effect of parental nutrition. However, higher LO levels (80% replacement of fish oil) in broodstock diets did not improved growth of 16 month-old offspring, in agreement with previous studies (Izquierdo et al., 2015). Thus, feeding broodstock with this high LO diet markedly reduced spawning quality, larval survival and larval and juvenile growth (Izquierdo et al., 2015), as a consequence the deleterious effects of very low n-3 HUFA levels in broodstock diets (Fernandez-Palacios et al., 2011). The present study demonstrated the persistence of these negative effects of early essential fatty acid deficiencies along offspring life. On the contrary, 60% FO substitution with LO in seabream broodstock diets did not negatively affected spawning quality or larval growth and produced 4 month-old juveniles that with a better ability to utilize low FM/FO diets (Izquierdo et al., 2015), in agreement with the present study.

The above-mentioned growth improvement in 16-month-old fish obtained from broodstock fed moderate LO levels and the reminder low FM/FO at 4-month-old, was also accompanied with an enhanced utilisation of the low FM/FO diet, as denoted by the better FCR, which could be related to the modulation of lipid and carbohydrates metabolism. There were not large differences in the proximate composition and fatty acid profiles of seabream liver and muscle, reflecting the profound effect of the diet, regardless the nutritional history of the different fish groups. Nevertheless, both broodstock diet and reminder diet, had a significant effect on some major fatty acids in liver such as 18:0, a terminal product of lipogenesis, and in the ratios 18:0/16:0 and, particularly, 18:1/16:1. Both 16:0 and 16:1 are substrates for elovl6, a key rate-limiting enzyme in the long-chain fatty acids elongation cycle and, therefore the ratios 18:0/16:0

and 18:1/16:1, are indicators of the activity of this enzyme. These results are in agreement with the hepatic *elovl6* expression, which was down-regulated by the increase in LO in the broodstock diets and was correlated inversely to the 16:0 contents in the liver and directly to the 18:0/16:0 and 18:1/16:1, denoting a significant post-transcriptional effect. Besides, LO increase in broodstock diets also increased the hepatic 18:0/18:1 ratios in the 16-month-old offspring.

These results are in agreement with the 16:0 reduction and 18:0/18:1 increase in mice models with *Elovl6* disruption, which showed a protection against high saturated fat diet-induced insulin resistance that lead to hepatosteatosis similar to that of wild-type mice (Matsuzaka and Shimano, 2009). This protection was related to the restoration of hepatic insulin receptor substrate-2, suppression of hepatic protein kinase C ε, and restoration of Akt phosphorylation (Matsuzaka and Shimano, 2009), overall indicating a better utilisation of dietary carbohydrates under conditions of high fat diet-induced insulin resistance. Indeed, insulin stimulates acetyl-CoA carboxylase that produces malonyl-CoA, which inhibits CPT-I activity and affect utilisation of fatty acids and glucose as substrates (Zammit, 1999). Thus, genes related to fatty acid oxidation, such as *Cpt1*, are down-regulated in mice with *Elovl6* disruption (Matsuzaka and Shimano, 2009), whereas up-regulation of *Cpt1* expression caused by intrauterine growth restriction increases the risk of type 2 diabetes in adulthood (Corbin, 2011). In agreement, in the present study, down-regulation of *elovl6* was correlated to down-regulation of *cpt1,* a rate limiting enzyme for fatty acid oxidation in mitochondria (Kolditz *et al.*, 2008). Moreover, increased LO in broodstock diet and increased plant protein and lipid sources in the 4-month-old reminder diet induced the down-regulation of *cpt1* in the gilthead sea bream offspring evidencing a long-term nutritional programming effect. A down-regulation of *cpt1b* gene expression was also found in the liver of juvenile rainbow trout by vitamin supplementation at first feeding showing that the nutritional interventions during the developmental plasticity (larval period) may provoke longer term effects later in life (Panserat *et al.*, 2017). *Cpt1* expression in liver of fish is down-regulated by the reduction of dietary PUFA, particularly LC-PUFA (Morash *et al.*, 2009; Xue *et al.*, 2015). Accordingly, *cpt1l* expression is also down-regulated in Atlantic salmon when dietary FO is substituted with VO (Jordal *et al.*, 2005; Torstensen *et al.*, 2010). *In vitro* studies in

rainbow trout hepatocytes showed that *pparα* and *cptI* are up-regulated by MUFA and down-regulated by EPA, among other fatty acids (Jordal *et al.*, 2005; Torstensen *et al.*, 2010). In the present study *cpt1b* expression was highly correlated to 18:1n-9 and negatively correlated to 20:5n-3 in liver. Besides, LO increase in broodstock diets significantly reduced the liver contents on 16:4n-3, an intermediate product of ß-oxidation of EPA. In gilthead sea bream offspring, *cptI* expression in liver negatively correlated *ppara,* suggesting that nutritional programing by LO reduces beta-oxidation in the mitochondria but not in the peroxisomes. In mammals, parental feeding with a high lipid diet lead to hypomethylation of four specific CpG dinucleotides in *PPARa* and the modification of the mRNA transcript in juvenile offspring (Lillycrop *et al.*, 2008). In gilthead seabream fed a low FM/FO diet, hepatic *ppara* is reportedly down-regulated, in association with retarded growth (Benedito-Palos *et al.*, 2016). In the present study, *ppara* in the offspring of HLv, fed a low FM/FO diet was not down-regulated and growth was even increased instead of reduced by the parental feeding with LO.

In previous studies, parental feeding of gilthead sea bream with increased substitution of FO with LO significantly up-regulated *fads2* in one-month-old offspring (S. Turkmen *et al.*, in prep.). Similarly, in Senegalese sole, parental nutritional history affects growth performance and the expression of *Δ4fad* and *elovl5* in the 2-month-old progeny (Morais *et al.*, 2014). However, in the present study, *fads2* of the 16-month-old fish did not show significant differences, which could be related to the strong influence of the very low FM/FO diets fed to the 16 month-old seabream. Indeed, *fads2* relative expression was high in all fish and up to 5.5 times higher than the values in gilthead sea bream juveniles fed commercial diets containing high levels of FM and FO (data not shown). This is in agreement with the up-regulation of *fads2* expression in fish fed reduced n-3 LC-PUFA diets rich in linolenic or LA (Vagner and Santigosa, 2011). Dietary changes did not affect *fads2* gene expression in Atlantic cod (*Gadus morhua*) either (Tocher *et al.*, 2006), reportedly related to the low dietary FO levels causing an up-regulation of this gene (Izquierdo *et al.*, 2008) or to a post-transcriptional regulation (Izquierdo *et al.*, 2008) as observed in other marine fish species (Geay *et al.*, 2010). Despite a slightly higher *fads2* expression in offspring of broodstock fed LO and the reminder diet, individual differences among fish belonging to the same treatment lead to

large variations with no significant differences among groups. Nevertheless, *fads2* expression in liver was correlated with hepatic contents in 18:4n-3, 18:3n-6, 20:5n-3, 22:6n-3 and 22:5n-6, intermediate and end products of desaturation activity by this enzyme (Tocher, 2003).

The down-regulation of *lpl* expression in the liver of offspring from broodstock fed feeds with high LO levels, especially in those fish that received a low FO/FM diet during juvenile stages, was correlated with reduced liver lipid contents, in agreement with the reduced lipid deposition associated to down-regulation of *lpl* expression in liver of gilthead sea bream in previous studies (Saera-Vila *et al.*, 2005). LPL is determinant of lipid fate deposition or catabolism (Leaver *et al.*, 2008). Thus, nutritional programing through regulation of different genes within the pathway of lipid metabolism including *lpl*, *elovl6* and *cpt-I*, may prepare the offspring for a better utilisation of low FM and FO diets, improving VO and VM utilisation and reducing the risk of hepatosteatosis described in gilthead sea bream fed these type of diets (Montero *et al.*, 1996). As occurs in mammals, nutritional signals through parental feeding may improve offspring fitness at later stages triggering a "predictive adaptive response" (Gluckman *et al.*, 2005). Thus, in gilthead sea bream, offspring of broodstock fed moderate LO levels and fed the low FM/FO diet during juvenile stages showed improved final body weight and feed utilisation.

To our knowledge, this is the first study that demonstrates the profound effects of n-3 LC-PUFA profiles in parental diets on long-term effects in fish offspring even later in life and in the verge of their first sexual maturation. In mammals, n-3 LC-PUFA supplementation in maternal diets reduces premature births (Cetin and Koletzko, 2008) and enhances immune health (Uauy *et al.*, 2009; Calder *et al.*, 2010), growth, development and pancreatic tissue morphometry in the offspring (Siemelink *et al.*, 2002). Besides, the nutritional programing effect of LC-PUFAs on parental diet and the epigenetic regulation of gene expression has been also demonstrated in mammals(Jaenisch and Bird, 2003), implying different epigenetic and physiological mechanisms including cell differentiation, neuro-hormonal regulation, etc. (Hyatt *et al.*, 2007), which have not been yet demonstrated in fish. The present study has also point out that nutritional programming through parental feeding interacts with the feeding

history during juveniles stages, since feeding a low FM/FO diet for only one month when fish were 4 months-old affected gene expression and fish performance later when fish were in the verge of reproduction, in agreement with studies in other vertebrates (Duque-Guimaraes and Ozanne, 2013). In summary, partial replacement of FO with LO in parental diets during gilthead sea bream reproduction induced long-term persistent effects on offspring transcription in selected genes that regulate energy metabolism in liver for a better utilisation of diets high in VO and VM. Moreover, these long-term effects on gene transcription are further enhanced by feeding the offspring juveniles with diets high in VO and VM, what improved growth and feed utilisation. Studies are underway to better understand the potential epigenetic, metabolic and molecular mechanisms involved in the metabolic conditioning of offspring through parental nutrition in gilthead sea bream.

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5.6 Author contributions

S.T. conducted all experiments, analysed and evaluated all biological, biochemical, molecular analysis. Molecular biology samples were analysed by S.T. with the supervision of M.J.Z. All trials were designed by S.T. D.M. and C.M.H., and M.I. supervised the entire work. L.R. was involved in the formulation and the preparation of the diets. The manuscript was written by S.T. and M.I.

Chapter 6

Broodstock selection and nutritional programming with ALA (18:3n-3) rich diet affects the growth performance of the offspring in gilthead sea bream

Submitted to Aquaculture journal as Turkmen *et. al.*,

Abstract:

The complete removal of fishmeal (FM) and fish oil (FO) is required to promote the sustainable development of aquaculture and for that, fast-growing high-quality fish that are fed without FM and FO are necessary. Early nutritional programming may allow the production of fish better adapted to utilize diets with vegetable meals (VM) and oils (VO). The main objective of this study was to research in the potential value of fatty acids as modulators of early nutritional programming in marine fish for a better utilization of VO/VM. For that purpose, gilthead sea bream (*Sparus aurata*) broodstock were fed four different replacement levels of FO by linseed oil (LO) and their effect on fecundity and spawn quality, egg composition, Δ-6-desaturase (*fads2*) gene expression, progeny growth performance and their growth response to a challenge with diets low in FO and FM, but high in VO and VM. The results showed that feeding gilthead sea bream broodstock with high LO diets had very long-term effects on the progeny. Thus, FO replacement by LO up to 80–100% in broodstock diets for gilthead sea bream not only reduced fecundity and spawn quality, but also growth of 45 dah and 4-month-old juveniles, as well as *fads2* gene expression. However, when the 4-month-old juveniles were fed with a low FM and FO diet, even those from broodstock fed only 60% replacement of FO by LO showed a higher growth and feed utilization than juveniles from parents fed FO. These results demonstrate the interesting potential of early nutritional programming of marine fish by broodstock feeding to improve long-term performance of the progeny. Further studies are being conducted to determine optimum nutrient levels in the broodstock diets and the molecular mechanisms implied to develop effective nutritional intervention strategies for this species.

Keywords: nutritional adaptation of offspring, long term effects of parental nutrition, broodstock selection, fatty acid desaturase (fads2), epigenetics in aquaculture

6.1 Introduction

Development of the aquaculture production is fundamental for the growing human population as fish being a high-quality protein and lipid source for human nutrition. According the latest available statistics by (FAO, 2018), aquaculture production continues its steady growth approximately at a rate of around 5-10% yearly during the last decade and was reached to 110 million tonnes by 2016. The issue of high utilization of quality products such of fishmeal (FM) and fish oil (FO) in aquafeeds has received considerable critical attention in aquaculture production during the last decades. Since these sources are capture-derived and fisheries production remains constant during the last decade cannot foster the sustainable development of the sector (Kaushik and Troell, 2010). Thus, in recent years, there has been an increasing interest in high or complete substation of FM and FO substation with more readily available terrestrial sources such as vegetable oils (VO), impacts negatively upon a range of different parameters such as reducing the fish growth and negatively influence the fish health (Izquierdo *et al.*, 2005; Montero and Izquierdo, 2010; Rosenlund *et al.*, 2010).

One of the main boundaries about the VO as a total replacer of FO are their fatty acid composition. While marine originated oils, namely FO, are rich sources of n-3 LC-PUFA, terrestrial oils especially VO are rarely having 20C or more fatty acids and totally lacking essential fatty acids for marine fish such as EPA and DHA (Orsavova *et al.*, 2015) -with the exception of genetically modified crops (Betancor *et al.*, 2016). However, VOs, are rich sources precursors of *de novo* biosynthesis of 18:3n-3 (ALA) and 18:2n-6 (LA) fatty acids to n-3 LC-PUFA and particularly linseed oil (LO) has a very high ALA content which is the primer substrate for the n-3 LC-PUFA biosynthesis for EPA and DHA in humans and fish (Orsavova *et al.*, 2015). In this synthesis pathway there are several elongation, desaturation and β-oxidation involved and particularly in fish species, there are differences in evolution of the synthesis capacity (Castro *et al.*, 2016; Monroig *et al.*, 2018). Therefore, maximizing the capacity of these pathways of a given species is of interest to better utilisation of VO oil sources in fish.

Recently, a considerable literature has grown up around the theme of possible channelling of fish to better utilise the desired feed ingredients by early nutritional

conditioning (Engrola *et al.*, 2018; Panserat *et al.*, 2019) known as early-life programming in mammals (Langley-Evans, 2015). Nutritional programming presumes that early environmental clues such as diet provide a tool to the organism to forecast the environmental challenges in later life and give opportunity to adjust its metabolism for a better adaptation thus, provides higher possibility for the survival and reproduction to the organism (Burdge and Lillycrop, 2010). Hence, this tool may have possible applications in the animal production sector, such as aquaculture, to channel the individuals to better utilization of key nutrients (Gotoh, 2015). In rodents, maternal dietary composition had influence on programming growth and glucose metabolism in the offspring (Siemelink *et al.*, 2002). Gilthead sea bream is a multi-batch spawner whose oligolectic eggs largely depend on their continuous intake of nutrients during reproduction (Fernández-Palacios *et al.*, 2011). Therefore, nutrient egg composition can be modified to some extent by the diets used during the spawning period (Fernández-Palacios *et al.*, 2011). Indeed, previous research in gilthead sea bream has established that feeds supplied during the spawning season plays a critical role in regulating the lipid metabolism especially fatty acid desaturation and elongation pathways such as fatty acyl desaturase 2 (*fads2*), fatty acid elongase 6 (*elovl6*), carnitina palmitoiltransferasa I (*cpt1*), together with some genes involved in lipid metabolism such as lipoprotein lipase (*lpl*) in liver thus triggering different phenotypes in the offspring for better utilisation of vegetable meal (VM)/VO based diets at 4-months-old (Izquierdo *et al.*, 2015)(Han et al., submitted) and even 16-months-old of age during the juvenile stages (Turkmen *et al.*, 2017). Additionally, the research showed some stress related genes are also regulated in the offspring obtained from the broodstock feeding with different FO to VO oil replacement ratios (Turkmen et al., submitted) in early larval stages. Besides, studies in different species such as Atlantic salmon, revealed that vegetable-based diets utilisation can be improved by early dietary interventions (Clarkson *et al.*, 2017) and liver transcriptome analysis revealed up-regulation of genes involved but not limited in oxidative phosphorylation, pyruvate metabolism, TCA cycle, glycolysis and fatty acid metabolism (Vera *et al.*, 2017). Thus, nutritional innervations during the developmental plasticity by different dietary fatty acids may regulate the lipid utilisation in later stages in fish.

In general, the research to date has tended to focus on the effects of global effects FO substitutions with VO thus reducing the n-3 LC-PUFA (n-3 series fatty acids with more than 20C atoms) and increasing the 18C precursors in the broodstock diets and its long term-effects on the progeny or DHA reduction in the diets (Fuiman and Perez, 2015). These studies being the first studies in its field, had promising results to improve the high VM/VO diets in the on-growing period. Nevertheless, it is not clear if these alternations are caused by the 18C precursor abundancy and/or n-3 LC-PUFA deficiency in the programming diet used in the spawning period.

On the other hand, in animal production genetic selection of the livestock gives opportunity to create population with economic production efficiency (Luiting, 1990). For instance, harvest weight sizes in aquaculture species such as 900% increase can be gained in trout harvest weight, which may have major impact on the aquaculture growth (Janssen *et al.*, 2017). Moreover, this selection programs can be also tailored to the present needs in aquaculture such as improvement of the novel and less costly feeds utilisation high in VM and VO. Indeed, a research conducted in rainbow trout (*Oncorhynchus mykiss*) has shown a single generation selection caused up to 35.3% body weight gain in fish fed 100% VM and VO oil diets free of marine sources (Le Boucher *et al.*, 2012). One of the approaches in the animal breeding programs are identifying the biological biomarkers for a certain expected outcome by identifying the novel genes with desirable characteristics or genetic selection (Cassar-Malek *et al.*, 2008). In this sense, *fads2* gene which codifies the delta-6-desaturase, the rate limiting step of the LC-PUFA biosynthesis, is a very strong candidate since its regulation in response to VO are well documented variety of fish species (Vagner and Santigosa, 2011) as well as in gilthead sea bream (Izquierdo *et al.*, 2008). While there are several successful studies on the effects of selection to decrease the deformity prevalence in gilthead sea bream (JUANMA's works?) Very recently, in parallel to the increasing utilisation of VO in aquafeeds and the environmental concerns about FO production, some research is started to explore Δ-6 desaturase capacity as a selection criterion in atlantic salmon (*Salmo salar*) to improve VO based diets (Lutfi, ISFNF 2018). However, studies focusing on selection of the individuals for the better utilisation of VM/VO in gilthead sea bream are scarce.

The experimental work presented here provides one of the first investigations into how combination of broodstock feeding with feeds in high levels of VO (high 18C diet) and selection of the broodstock by their *fads2* expression in fish and its effects on the spawning quality, growth in larval period, the response of the juvenile fish in terms of growth at 6 months of age if challenged with a very low FM/FO (5% FM and 3%FO) diet. Moreover, the study investigated the effects the above-mentioned parameter on the fillet muscle fatty acid composition in juveniles to give a valuable information for the costumer perspective.

6.2 Materials and Methods

6.2.1 Broodstock selection and spawning

A total of 70 gilthead sea bream broodstock fish including 42 females and 28 males were used for the selection of individuals for the current investigation. These individuals were fed with high VO diet (Table 6-1) for 1% biomass ratio at 08:00 and 14:00 h except on Sundays for 1 month. After the feeding period, whole blood was taken from the caudal vein of the brood fish for the selection of the individuals. Prior to measurements all fish were anesthetized with 10 ppm clove oil/methanol (50:50) in sea water. 2 mL blood was taken with 2 mL sterile syringes (Terumo Europe NV, Leuven, Belgium) and transferred to 2.5 mL K 3 EDTA tubes (L.P. Italiana, Milan, Italy). Whole blood samples were always kept on ice during the sampling and were immediately centrifuged at 10000 rpm, 4°C for 20 minutes. Plasma were separated, and erythrocytes were snap frozen with liquid nitrogen and kept at -80°C until RNA extraction. RNA extraction and purification are explained more in detail in molecular studies section.

6.2.2 Spawning quality parameters

Spawning quality was determined before and after feeding the experimental diets. At the beginning of the trial, mean body weight and total length for females and males was 2.21±0.37 kg and 1.67±0.30 kg, and 43.94±1.91 cm and 40.71±3.66 cm, respectively. Fishes with high and low *fads2* expression were randomly assigned to experimental tanks using 1 female and 1 male ratio per tank. After placing the brood fish in the experimental tanks, fish were fed with commercial diets, and spawning quality monitored daily. Once similar spawning quality parameters observed between the tanks

during the acclimation period (P>0.05, data not shown), tanks were randomly assigned one of the experimental groups.

Experiment groups were;

-**FHD**: fed with 100%FO diet (F) and high *fads2* expression broodfish (HD)

-**FLD**: fed with 100%FO diet (F) and low *fads2* expression broodfish (LD)

-**VHD**: fed with 30%FO-70%VO diet (V) and high *fads2* expression broodfish (HD)

-**FLD**: fed with 30%FO-70%VO diet (V) and low *fads2* expression broodfish (LD)

Table 6-1 | Main ingredients*, energy, protein and % total fatty acids contents of diets used in conditioning test

Main Ingredients (%)	%	Proximate Composition	(% dry matter)
Fish meal SA[1] 68 super prime	5.00	Crude lipids	21.7
Fish meal alternative protein sources[2]	54.50	Crude protein	45.1
Rapeseed meal cake	11.30	Moisture	9.0
Wheat	6.89	Ash	5.4
Fish oil SA[1]	3.00		
Vegetable oil mix[3]	13.00	Gross Energy (MJ/kg, as is)	22.5
% total fatty acids		% total fatty acids	
14:0	6.6	18:3n-3	11.8
14:1n-5	0.1	18:4n-3	0.4
15:0	0.1	18:4n-1	0.0
16:0ISO	0.0	20:0	0.4
16:0	12.3	20:1n-9	0.0
16:1n-7	2.1	20:1n-7	1.0
16:1n-5	0.1	20:1n-5	0.1
16:2n-4	0.2	20:2n-9	0.0
17:0	0.3	20:2n-6	0.1
16:3n-4	0.1	20:3n-9	0.0
16:3n-3	0.0	20:3n-6	0.0
16:3n-1	0.0	20:4n-6	0.2
16:4n-3	0.4	20:3n-3	0.0
18:0	3.2	20:4n-3	0.1
18:1n-9	32.3	20:5n-3	2.5
18:1n-7	2.3	22:1n-11	0.1
18:1n-5	0.0	22:1n-9	0.3
18:2n-9	0.0	22:4n-6	0.0
18:2n-6	20.3	22:5n-6	0.1
18:2n-4	0.1	22:5n-3	0.3
18:3n-6	0.1	22:6n-3	1.7
18:3n-4	0.0		

* Please see Torrecillas *et al.*, (diet code 5FM/3FO for the complete list of feed ingredients.
1South American, Superprime (Feed Service, Bremen, Germany).
2Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
3Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

Formulation of the diets used in broodstock feeding during the nutritional conditioning period were given in Table 6-2. The spawning quality parameters were determined using eggs those were collected daily from each tank at around 08:00h and concentrated in 5 L beakers. Immediately, eggs were transferred to the laboratory and aeration supplied to each to ensure the mixing of the eggs through the water column. 5 mL sample was taken by using graduated glass pipet and transferred to a Bogorov chamber. Eggs were counted and observed under a binocular microscope (Leica Microsystems, Wetzlar, Germany) in five replicates to calculate: the total number of eggs, the percentage of fertilised eggs and determine the morphological characteristics. Egg viability rate, determined as the percentage of morphologically normal eggs at the morula stage and described as transparent, perfectly spherical with clear, symmetrical early cleavages (Fernández-Palacios *et al*., 2011). After that, some egg randomly was taken and transferred to 96 well ELISA microplates using a micropipette (0.7 mL of seawater and one egg per well). Plates were observed under a binocular microscope to ensure there was a single fertilized egg at each well. These eggs were kept temperature-controlled refrigerator to keep the temperature at constant at 23 °C. By observing the egg from these ELISA plates after 24 and 72 hours under a binocular microscope, hatching rate and survival after three dah were calculated as percentages. With these percentage values, the total number of fertilized, viable and hatched eggs and larvae survived after 3dah calculated per female/kg basis.

6.2.3 Larval rearing and growth

After the one month of feeding with experimental diets, eggs were collected and distributed to 500 L tanks for mass production at a density of 100 eggs/L from each broodstock groups. Water renewal in the tanks were progressively increased from 10 to 40% per h until 46 dah. Water was continuously aerated (125 mL/min). Larvae will be reared under natural photoperiod and living phytoplankton (*Nannochloropsis sp.*) (250 ± 100 x 103 cells per mL) was added to the rearing tanks. From 3-32 dah, larvae were fed twice a day rotifers (*Brachionus plicatilis*) at 10 rot/mL enriched with commercial emulsions. From 15-17 dah, *Artemia sp.* enriched with commercial emulsions will be added 3 times a day. From 20 dah, larvae was fed commercial diets (Gemma Micro,

Skretting, France). Water was continuously aerated (125 mL min^{-1}) attaining 6.1±0.4 ppm dissolved O_2.

Table 6-2 | Formulation, main ingredients and biochemical composition of the nutritional programming diets supplied 1 month before egg collection for larval rearing

Raw material (%)	100% FO (F)	30% FO-70% VO (V)
Meals from marine sources[1]	50.0	50.0
Sunflower cake	13.2	13.2
Soya cake[2]	10.0	10.0
Fish oil[3]	8.0	2.4
Linseed oil[4]	-	5.6
Wheat	9.9	9.9
Corn gluten 60	7.0	7.0
Drying / wetting	0.9	0.9
Vitamin & mineral premix[5]	1.0	1.0
Vitamin E powder (50%)	0.1	0.1
Biochemical composition (% of dry matter)		
Moisture	9.1	8.8
Protein (crude)	56.3	56.1
Lipids (crude)	17.2	17.1
Ash	8.6	8.5
Energy - gross (MJ kg^{-1})	21.2	21.2
Fatty acids (% of total fatty acids)		
16:1n-7	7.1	4.3
18:2n-6	5.6	9.9
18:3n-3	0.9	16.3
20:1n-7	12.4	6.8
20:4n-6	0.4	0.3
20:5n-3	6.3	4.8
22:1n-11	15.7	8.6
22:6n-3	7.1	6.0

1 Contains Fish meal NA LT 70, Fish meal SA 68, Feed Service Bremen, Germany.
2 48 Hi Pro Solvent Extra. Svane Shipping, Denmark
3 South American fish oil, LDN Fish Oil, Denmark
4 Ch. Daudruy, France
5 Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany)

Average water temperature and pH along the trial were 21.1±0.4°C and 7.0±0.6, respectively. Water quality was daily monitored by dissolved O_2 and pH, and since none of these parameters changed along the trial, no further parameters such as nitrites or nitrates contents were studied. At 3, 15 and 30 dah, growth was determined by measuring total length of 30 anesthetized larvae using a profile projector (V-12A Nikon; Nikon, Tokyo, Japan). Additionally, at 30 dah, around 100 mg of unfed sea bream larvae were collected and conserved in 500 µL of RNA Later (Sigma-Aldrich, Madrid, Spain) overnight at 4°C, then RNA later was removed and samples kept at -80°C until RNA extraction. RNA extraction methods are detailed in molecular studies section. At 46 dah, the whole population were transferred to 10,000 L tanks.

6.2.4 Juvenile feeding challenge experiment

All the fishes obtained from different broodstock groups kept in different tanks but similar conditions in triplicates. Fishes were fed with the same commercial diets through the grow-out period until they reach 6-months-of-age. Then fishes were assigned to experimental tanks in triplicates for each broodstock group. Thus, twelve 500 L capacity tanks were used for the juvenile feeding challenge experiment. All fish were anesthetized with 10 ppm clove oil: methanol (50:50) in sea water prior to measurements for the initial and final samplings. Fishes were fasted for 24 hours then individually weighted, total lengths were measured, and fishes were assigned to each experimental tank. Fishes were fed with experimental diet (Table 6-3). twice a day at 08:00 and 14:00h for 60 days, except for Sundays. Feed consumption was daily recorded. Growth was determined by measuring wet body weight after 24 h starvation. Prior to measurements all fish were anesthetized with 10 ppm clove oil: methanol (50:50) in sea water. Graphical demonstration of the experiment was presented in (Figure 6-1). Calculations of the growth parameters were calculated using the following equations; feed conversion ratio (FCR) = (total weight of consumed feed, g) / (final biomass – initial biomass, g) and % Weight gain (WG) = (final biomass – initial biomass)/initial biomass × 100.

Table 6-3 l Formulation, main ingredients and biochemical composition of the nutritional programming diets supplied 1 month before egg collection for larval rearing

Main Ingredients	%	Proximate Composition	(% dry matter)
Fish meal SA[1] 68 super prime	5.0	Crude lipids	21.8
Fish meal alternative protein sources[2]	54.5	Crude protein	57.2
Rapeseed meal cake	11.3	Moisture	6.5
Wheat	6.9	Ash	6.7
Fish oil SA[1]	3.0		
Vegetable oil mix[3]	13.0	Gross Energy (MJ/kg, as is)	22.5
% total fatty acids		% total fatty acids	
14:0	4.69	18:3n-4	0.04
14:1n-5	0.17	18:3n-3	14.86
15:0	0.28	18:4n-3	0.91
16:0	13.67	20:0	0.21
16:1n-7	4.50	20:1n-9	0.37
16:1n-5	0.09	20:1n-7	7.25
16:2n-6	0.25	20:1n-5	0.37
17:0	0.13	20:2n-6	0.16
16:3n-4	0.14	20:4n-6	0.31
16:3n-3	0.14	20:3n-3	0.09
16:3n-1	0.04	20:4n-3	0.19
16:4n-3	0.22	20:5n-3	3.81
18:0	3.23	22:1n-11	9.35
18:1n-9	15.75	22:1n-9	1.00
18:1n-7	2.46	22:4n-6	0.04
18:1n-5	0.23	22:5n-6	0.03
18:2n-6	9.84	22:5n-3	0.28
18:2n-4	0.05	22:6n-3	4.49
18:3n-6	0.08		

* Please see Torrecillas *et al.*, (diet code 5FM/3FO for the complete list of feed ingredients.

1. South American, Superprime (Feed Service, Bremen, Germany).

2. Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).

3. Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

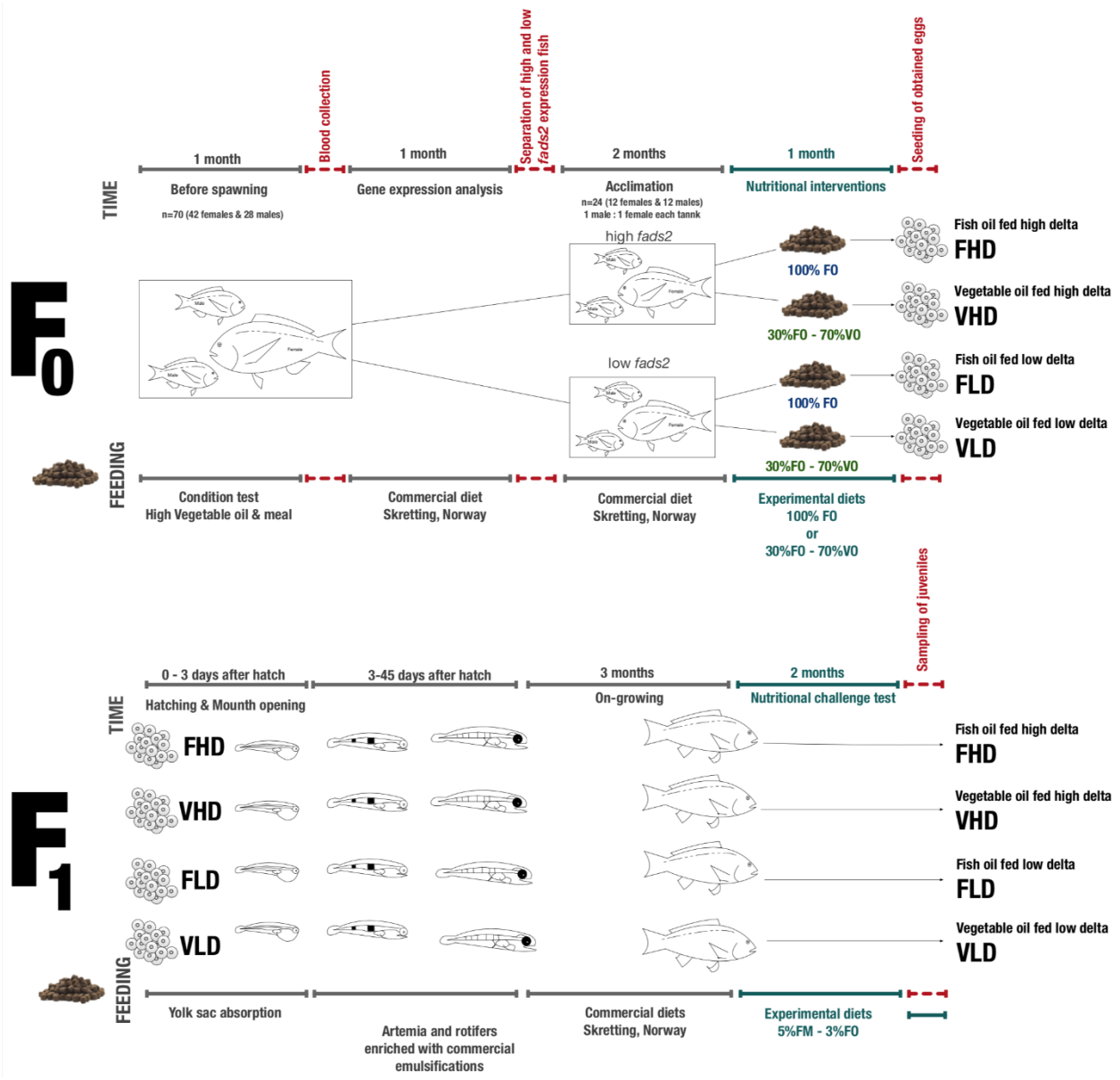

Figure 6-1 | Experimental design and sampling points, all groups were tested in triplicates. Sampling points were shown as , and experimental periods when fish were fed with different diets are shown as

6.2.5 Molecular studies

Samples were kept in the room temperature until samples were thawed completely. For each 200 µl of blood cells 1 mL of TRI Reagent (Sigma-Aldrich, Misuri, USA) were added into 2 mL Eppendorf tubes. To each tube, four pieces of 1mm diameter zirconium glass beads added and homogenized using TissueLyzer-II (Qiagen, Hilden, Germany) for 60 seconds with a frequency of 30/s. 250 µL chloroform was added to homogenized samples and then centrifuged at 12000 G for 15 min at 4 °C to phase separation. The transparent upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column where total RNA bonded to a membrane. After that RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen) with the protocol supplied by the manufacturer. Real-time quantitative PCR was performed in an iQ5 Multi-colour Real-Time PCR detection system (Bio-Rad) using *acbt* as the housekeeping gene in a final volume of 15 µl/reaction well and with 100ng of total RNA reverse-transcribed to complementary DNA (cDNA). Samples, housekeeping genes, cDNA template and reaction blanks were analysed in duplicates. Primer efficiency was tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:100 and 1:1000). Sequence of the primers used in this study were, *acbt* (GeneBank access no: X89920) 5'-3' (F): TCT GTC TGG ATC GGA GGC TC, 5'-3' (R): AAG CAT TTG CGG TGG ACG, *fads2* (GeneBank access no: AY055749) 5'-3' (F): CGA GAG CCA CAG CAG CAG GGA, 5'-3' (R): CGG CCT GCG CCT GAG CAG TT. Ninety-six-well PCR plate was used to analyse each gene, primer efficiency and blank samples (Multiplate; Bio-Rad). Melting-curve analysis was performed, and amplification of a single product was confirmed after each run. Fold expression of each gene was determined by delta-delta CT method ($2^{\Delta\Delta CT}$)(Livak and Schmittgen, 2001). PCR efficiencies were similar, and no efficiency correction was required (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

6.2.6 Statistical analysis

The results were expressed as the mean ± standard deviation (n=3) if not otherwise stated in the tables and figures. The data were compared statistically using the analysis of variance (ANOVA), at a significant level of 5%. All variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the

Levene tests, respectively (Sokal and Rohlf, 1969). If significant differences were detected with ANOVA, means were compared by a student t-test. All data were analysed using IBM SPSS v23.0.0.2 for Mac (IBM SPSS Inc. Chicago, IL, USA).

6.3 Results

6.3.1 Broodstock selection, spawning quality and eggs

Broodstock fish showed very high difference in expression of *fads2* gene after feeding conditioning diet (VM and VO diet) among individuals. The average fold gene expression was 7.1±13.6 being the max 61.6 and the minimum 0.02. No linear correlation found between the fish weight and *fads2* expression among individuals (R=0.0061). There were no differences in total number, fertilized, viable, hatched eggs and survival of 3 dah larvae if fed with same commercial diets (49023±1195, 46339±1862, 33990±2513, 31899±3092, 22960±2114,respectively)(P>0.05). After feeding the broodstock with a FM-FO or FM-VO diet during one month, total number of produced eggs and survival of 3 dah larvae were lower in low *fads2* expression groups regardless of the diet fed (37% total number of eggs and 10.2% more 3 dah larvae in high *fads2* groups) (P<0.05)(Figure 6-2). Same tendency was observed for the rest of the tested parameters such as fertilized, viable, hatched eggs without significance difference among groups (P>0.05)(Figure 6-2).

Obtained eggs were analysed for their biochemical composition after the acclimation period. In addition, feeding the selected fishes for one month with the experimental diets were not affected the biochemical composition of the eggs obtained (P>0.05) (Table 6-4). Selection, diet nor the interaction between these parameters showed significant effects on biochemical composition of the eggs (P>0.05) (Table 6-4). However, fatty acid composition was altered after one month of feeding period, FO substitution by VO significantly increased 18:3n-3 and 18:2n-6, regardless of the selection groups (P>0.05)(Table 6-5), those fatty acids those were abundant in VO diet and reflecting the diet's fatty acid composition (Table 6-5). From the saturated fatty acids 14:0 and 17:0 were lower in VO fed groups (P<0.05), while the other saturated fatty acids such as 15:0, 16:0, 18:0 and 20:0 were similar among all the groups (P>0.05) (Table 6-5).

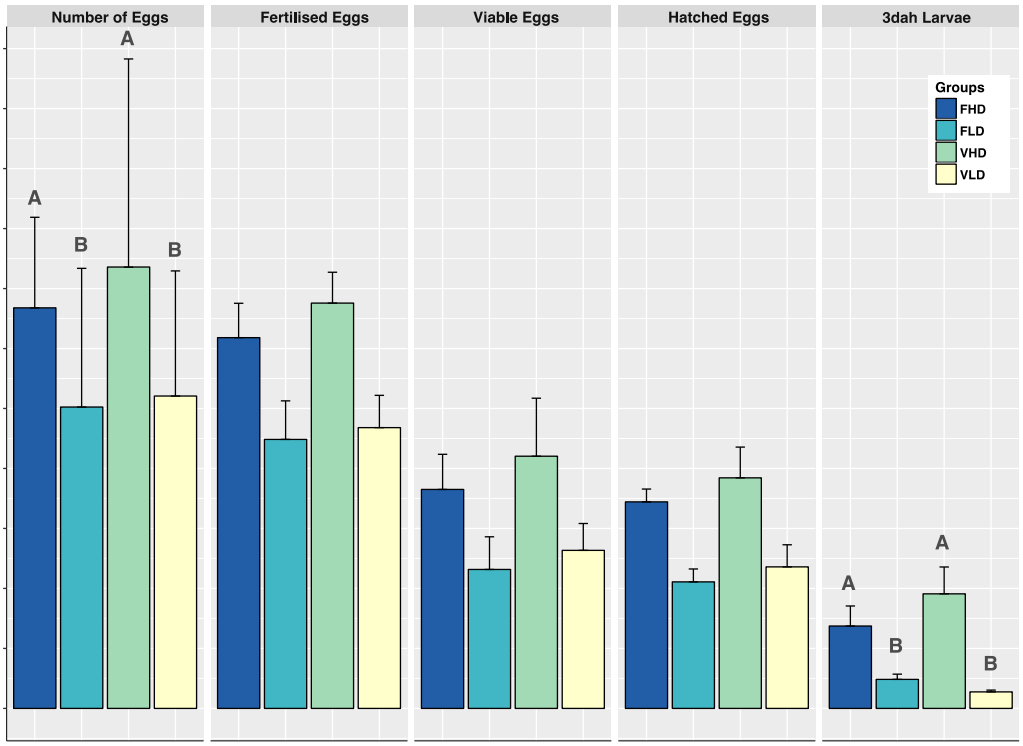

Figure 6-2 | Spawning parameters of gilthead seabream broodstock fed with either 100%FO (black bars – F groups) or 70%VO 30%FO (grey bars – V groups) for 1 months. Broodstock fishes were separated according high (darker colour bars) or low (lighter colour bars) fatty acyl desaturase 2 gene expression levels in blood. Differential letters denote differences between groups (P<0.05). Data presented as mean±SD (n=3)

Table 6-4 | Proximate composition of eggs obtained from gilthead sea bream after feeding broodstock diets with substitution of FO by LO (% DM, mean ± SD, n = 3 (one pool of eggs from all the spawns per tank)

	Crude protein	Crude lipids	Ash
Groups	**%dry weight**		
FHD	69.83±2.67	22.39±5.10	16.27±1.43
FLD	63.29±2.86	23.26±2.54	19.44±3.55
VHD	69.34±0.84	25.97±1.60	17.82±0.54
VLD	68.16±3.23	26.89±0.21	13.25±0.62
Two-way ANOVA*			
Selection	ns	ns	ns
Diet	ns	ns	ns
SxD	ns	ns	ns

*All values are mean ± SD (n=3). No significant differences were shown as ns (P>0.05). No significant differences found among the groups.

Also similar to the diet fatty acid compositions monoenoic fatty acids such as 14:1n-7, 14:1n-5, 16:1n-7, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5 and 20:1n-11 were lower in VO fed groups (Table 6-5). On the other hand, 18:3n-3 – first substrate for the n-3 LC-PUFA biosynthesis to EPA and DHA- was up to 4 times higher in eggs obtained from VO fed groups ($P<0.05$) (Table 6-5), these difference was low in comparation to diets whereas VO diet had more than 18 times higher 18:3n-3. Besides, 18:2n-6 in the eggs -another substrate for n-3 LC-PUFA biosynthesis and rich in vegetable oils- was similar ($P>0.05$) (Table 6-5). In general, diet fatty acid compositions were correlated with the egg fatty acid composition. For the n-3 LC-PUFA biosynthesis pathway, there was an increase in only 18:3n-3 substrate in the egg fatty composition ($P<0.05$)(Table 6-5) while 18:2n-6 content were similar ($P>0.05$)(Table 6-5). Moreover, products of n-3 LC-PUFA biosynthesis such as 20:4n-6, 20:5n-3 and 22:6n-3 were also similar in the eggs ($P>0.05$) (Table 6-5).

Table 6-5 l Fatty acid composition of eggs obtained from gilthead sea bream after feeding broodstock diets with substitution of FO by LO (% total fatty acids, mean ± SD, n = 3 (one pool of eggs from all the spawns per tank)

					Two-way ANOVA		
% of total fatty acids	FHD	FLD	VHD	VLD	(S)	(D)	DxS
14:0	4.24±0.01^{Ax}	3.44±0.08^{B}	2.83±0.48^{y}	3.16±0.55		**	*
14:1n-7	0.05±0.00^{Ax}	0.04±0.01^{B}	0.02±0.01^{y}	0.03±0.01		**	*
14:1n-5	0.14±0.01^{Ax}	0.11±0.00^{B}	0.09±0.01^{y}	0.10±0.02		**	*
15:0	0.31±0.00	0.30±0.03	0.27±0.03	0.29±0.03			
15:1n-5	0.04±0.00^{Ax}	0.03±0.00^{B}	0.03±0.00^{y}	0.03±0.00			*
16:0ISO	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.01			
16:0	18.08±0.74	21.37±2.97	19.73±2.41	18.83±1.59			
16:1n-7	6.31±0.02^{x}	5.79±0.28^{x}	4.75±0.49^{y}	4.77±0.21^{y}		**	
16:1n-5	0.09±0.01	0.10±0.01	0.09±0.01	0.09±0.01			
16:2n-6	0.01±0.00	0.00±0.00	0.00±0.00	0.00±0.00			
16:2n-4	0.25±0.00^{Ax}	0.18±0.03^{B}	0.16±0.04^{y}	0.18±0.02		*	*
17:0	0.18±0.00^{Ax}	0.15±0.01^{B}	0.14±0.02^{y}	0.13±0.01	*	**	
16:3n-4	0.20±0.00	0.23±0.02^{x}	0.20±0.02	0.19±0.00^{y}		*	*
16:3n-3	0.12±0.01	0.10±0.01	0.09±0.02	0.10±0.02			
16:3n-1	0.06±0.00^{y}	0.09±0.02	0.08±0.01^{x}	0.08±0.01			
16:4n-3	0.07±0.03	0.07±0.02	0.06±0.01	0.06±0.00			
16:4n-1	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00			
18:0	3.67±0.24	3.98±0.69	4.03±0.62	4.03±0.58			
18:1n-9	19.67±0.63^{B}	22.88±1.71^{A}	21.25±1.98	20.51±0.67			*
18:1n-7	3.24±0.16^{x}	2.95±0.10	2.75±0.26^{y}	2.71±0.19		**	
18:1n-5	0.26±0.05^{x}	0.19±0.03	0.15±0.04^{y}	0.16±0.04		*	
18:2n-9	0.27±0.01^{x}	0.21±0.04	0.19±0.04^{y}	0.22±0.08			
18:2n-6	9.39±0.91	10.83±0.91	11.45±0.97	11.55±0.45		*	
18:2n-4	0.11±0.00	0.11±0.02	0.11±0.01^{A}	0.10±0.00^{B}			
18:3n-6	0.44±0.01^{A}	0.26±0.05^{B}	0.27±0.09	0.32±0.08			*
18:3n-4	0.12±0.01^{x}	0.11±0.01	0.10±0.00^{y}	0.10±0.02		*	
18:3n-3	1.82±0.64	1.68±0.40	5.49±2.70	7.28±3.36		**	
18:3n-1	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00			
18:4n-3	0.92±0.05	0.49±0.24	0.50±0.28	0.58±0.20			
18:4n-1	0.10±0.01^{Ax}	0.07±0.01^{B}	0.07±0.00^{y}	0.07±0.01	*	**	*
20:0	0.09±0.01	0.10±0.04	0.11±0.04	0.08±0.01			
20:1n-9	0.40±0.09^{Ax}	0.22±0.04^{B}	0.17±0.06^{y}	0.21±0.09		*	*
20:1n-7	3.16±0.68^{Ax}	1.52±0.36^{B}	1.12±0.36^{y}	1.36±0.54	*	**	*
20:1n-5	0.26±0.04^{Ax}	0.17±0.02^{B}	0.14±0.03^{y}	0.15±0.04		**	*
20:2n-9	0.08±0.01	0.08±0.01	0.07±0.02	0.07±0.04			
20:2n-6	0.31±0.02	0.38±0.05	0.32±0.05	0.30±0.02			
20:3n-9	0.03±0.00	0.02±0.00	0.02±0.00	0.02±0.00			
20:3n-6	0.16±0.02	0.14±0.01	0.12±0.01	0.13±0.03			
20:4n-6	0.61±0.02	0.62±0.06	0.63±0.04	0.61±0.10			
20:3n-3	0.16±0.01^{y}	0.17±0.01	0.27±0.07^{x}	0.29±0.07		**	
20:4n-3	0.62±0.08^{x}	0.46±0.10	0.43±0.07^{y}	0.44±0.05		*	
20:5n-3	5.54±0.65	3.93±1.28	4.00±1.12	4.09±0.39			
22:1n-11	1.23±0.30^{Ay}	0.46±0.13^{B}	0.35±0.16^{x}	0.51±0.29		*	**
22:1n-9	0.32±0.10	0.20±0.04	0.15±0.05	0.16±0.10		*	
22:4n-6	0.06±0.01	0.05±0.00	0.05±0.00	0.05±0.01			
22:5n-6	0.05±0.01	0.04±0.00^{x}	0.03±0.01	0.03±0.00^{y}		*	
22:5n-3	2.04±0.13	1.94±0.20	1.91±0.37	1.83±0.64			
22:6n-3	14.67±0.19	13.70±2.20	15.14±3.69	13.92±4.17			

†S refers broodstock selection, capital letters denote differences between selection groups fed with the same diet during the spawning period, D refers broodstock diet, lowercase letters denote differences among differentially fed broodstock groups during the spawning period with similar fads2 expression. DxS means interaction of these two parameters. *p<0.05, **p<0.01.

6.3.2 Larval growth

Despite the fact that larvae were kept in similar conditions and fed with the same diets, the growth were higher in FHD group in comparison with VHD group at 3 dah ($P<0.05$) (Figure 6-3 | Length (mm) of gilthead seabream larvae obtained from broodstock fed with either 100%FO (FHD, FLD) or 70%VO 30%FO (VHD, VLD) for 1 months. * represents days with significant difference between groups. Data presented as mean±SD (n=3)). However, growth of these groups was similar at 15 dah and 30 dah ($P>0.05$)(Figure 6-3). At 15 dah, among the progeny obtained from F fed broodstock FLD had higher length than FHD ($P<0.05$). In LD groups, VLD groups was smaller in comparison to FLD group ($P<0.05$). There were no differences in growth at 30dah ($P>0.05$)(Figure 6-3).

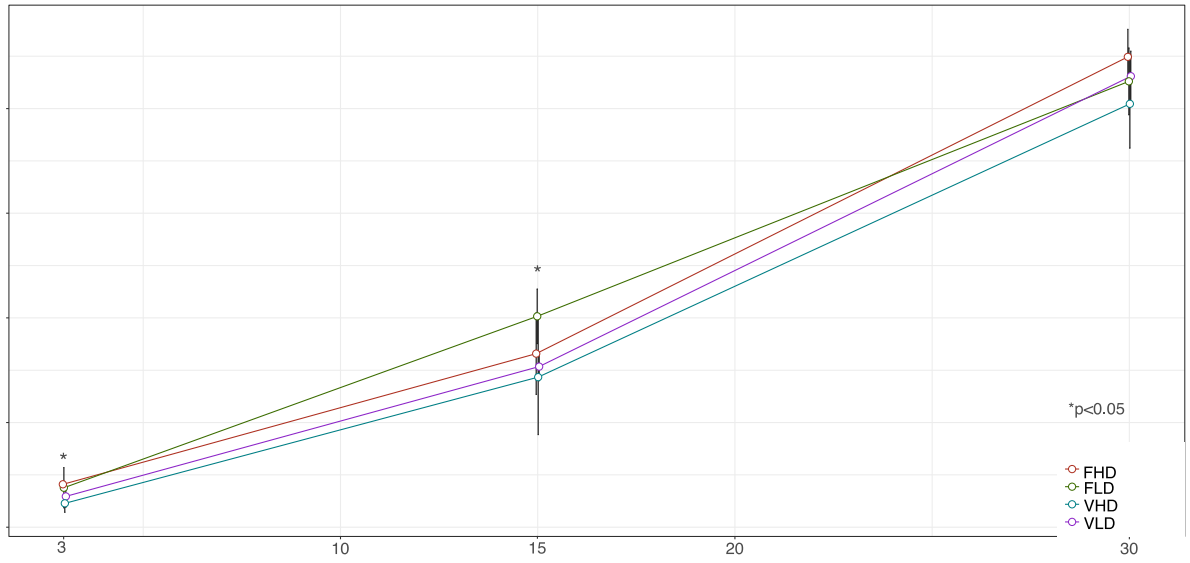

Figure 6-3 | Length (mm) of gilthead seabream larvae obtained from broodstock fed with either 100%FO (FHD, FLD) or 70%VO 30%FO (VHD, VLD) for 1 months. * represents days with significant difference between groups. Data presented as mean±SD (n=3)

6.3.3 Juvenile challenge test

There was no difference in the initial weights of the experimental groups ($P>0.05$) (FHD: 23.4±0.1, FLD:22.1±0.1, VHD:23.8±0.4 and VLD: 23.7±0.1). After feeding fish for 60 days with high VM and VO diet (Table 6-3), FHD group reached the highest final weight (47.4±0.8 g) and thus the highest weight gain (99.7±7.3%) (Figure 6-4). % final weight gain results showed selection of the broodstock had a higher effect on the weight gain ($P<0.001$) while diet ($P<0.01$), and interaction between these two parameters

(P>0.05) also found to be significant effects. Thus, selection of high *fads2* broodstock improved the weight gain regardless of the broodstock fed FO or VO diet (Figure 6-4). In addition, progenies obtained from broodstock selected for high *fads2* expression and fed with F diet (FHD), showed higher growth than progenies obtained from broodstock selected for high *fads2* expression and fed with V diet (VHD)(P<0.01) (Figure 6-4).

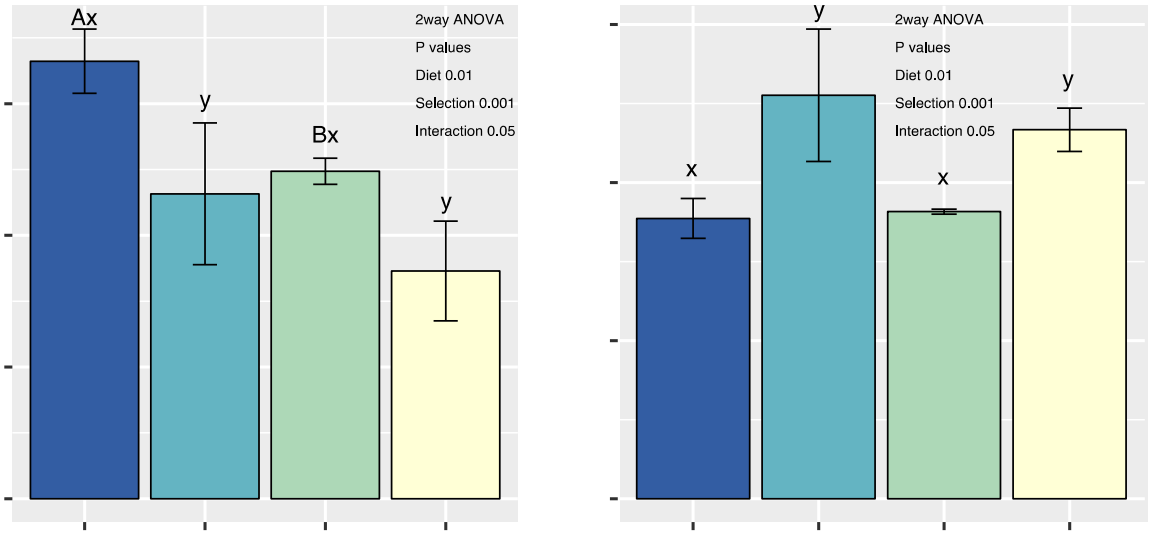

Figure 6-4 | Weight gain (%) and food conversion ratio FCR of gilthead sea bream juveniles from broodstock fed diets with fish oil (F) or 70% substation by linseed oil (V) after 60 days of a nutritional challenge with 5%FM and 3%FO diets (n=3, error bars=SD)

On the other hand, weight gain of the progenies obtained from broodstock selected for low *fads2* expression and fed with F diet (FLD) or V diet (VLD) were similar (P>0.05) (Figure 6-4). In summary, VO inclusion and increasing 18C fatty acids in the broodstock diets resulted lower weight gains in progenies if only selected for high *fads2* but not low *fads2* (P<0.05) (Figure 6-4). There was no difference in feed intake of the fishes (P>0.05) (data not shown). It has been also observed that the FCR values of the fishes coming from high *fads2* expression selected broodstock (HD groups) was lower regardless of the feed they receive during the spawning (P<0.05) (Figure 6-4). On the contrary, FCR values were significantly higher in progenies coming from low *fads2* selected broodstock, again broodstock diet had no significant effect on progenies FCR values (P<0.05) (Figure 6-4).

6.3.4 Fillet fatty acid profiles

Feeding the selected and nutritionally programmed fish through the broodstock nutrition affected the muscle fatty acid profiles of the fish, especially broodstock diet showed higher effect on the fatty acid profiles such as total saturated, monounsaturated and n-6 PUFA (P<0.05) while total n-3 PUFA contents were similar (Table 6-7). Total of the saturated fatty acids were higher in VLD group than VHD (P<0.05) while VHD had higher saturated fatty acids than the FHD (P<0.05). From the saturated fatty acids 14:0 and 16:0 were higher in VLD group than the VHD and FLD (P>0.05), thus two-way ANOVA showed significant differences on both selection (P<0.05), broodstock diet (P<0.01) and the interaction between these parameters (P<0.01)(Table 6-7).

Table 6-6 l Biochemical composition of muscle tissue (%dry weight) after 2 months feeding a very low fish meal (5%) and fish oil (3%) diet in 6 months-old gilthead seabream (*Sparus aurata*) originated from broodstock fed linseed oil in replacement of fish oil: 0% (F), 70% (V), (mean±SD, n=3) and selected either high *fads2* expression (HD) or low *fads2* expression (LD)

	Crude protein	Crude lipids	Ash
Groups	%dry weight		
FHD	67.09±6.40	26.62±5.45	1.51±0.09
FLD	65.83±3.28	24.25±1.86	1.33±0.19
VHD	69.53±3.55	27.21±1.27	1.50±0.02
VLD	67.44±2.48	24.96±3.27	1.69±0.19
Two-way ANOVA*			
Selection	ns	ns	ns
Diet	ns	ns	ns
SxD	ns	ns	ns

*All values are mean ± SD (n=3). No significant differences were shown as ns (P>0.05), and table shows P values of the two-way ANOVA analysis. Capital letters indicate differences effects of selection in the same feeding groups while small letter indicate effects of feeding in the same selection groups.

In addition, if LD groups compared 18:0 fatty acid were lower in VLD than the FLD group (P<0.05)(Table 6-7). Total monounsaturated fatty acids were affected by the diet in LD groups and was higher in VLD in comparation to the FLD group (P<0.05). n-6 PUFA were changed in response to the broodstock diet (P<0.05) and the interaction between broodstock diet and selection (P<0.1). 18:2n-6 was higher in VLD groups if compared with FLD (P<0.05) however, there was a clear tendency of 18:2n-6 accumulation in V fed broodstock progenies than the F fed broodstock (Table 6-7). The same tendency and

differences were observed in 18:3n-3, broodstock diet caused a tendency to accumulate more 18:3n-3 in V groups than the F fed broodstock while the accumulation was significantly higher in VLD than the FLD ($P<0.05$). On the other hand, there was no differences in 20:5-3 and 22:6n-3 and total n-3 PUFA content among the experimental groups ($P>0.05$)(Table 6-7). In addition, there were no differences in total PUFA content of the progenies obtained from broodstock selected for the fads2 expression and fed with either V and F diets ($P>0.05$).

Table 6-7 l Fatty acid composition of muscles (mg/100g of tissue) after challenge test diets with substitution of FO by LO (% total fatty acids, mean ± SD, n = 3 (one pool of eggs from all the spawns per tank)

	FHD		FLD		VHD		VLD				
Lipids (%)	26.62±5.45		24.25±1.86		27.21±1.27		24.96±3.27		Two-way ANOVA †		
Fatty acids (mg/100g)									S	D	DxS
14:0	174±88		98±33	y	B172±32	B	413±43	Ax	**	***	***
15:0	83±57		17±6		32±18		41±3				*
16:0	849±364		951±170	y	1885±175	B	2936±194	Ax	***	***	**
17:0	76±54		29±19		30±15		31±10				
18:0	359±77		380±55	y	668±138		677±29	x		***	
20:0	88±36		43±4		53±0		51±7				
Σ saturated	**1587±154**	**y**	**1715±12**		**2742±238**	**Bx**	**4273±97**	**A**	***	***	***
16:1n-9	77±50	A	4±2	B	8±4		10±1		*	*	*
16:1n-7	171±52	y	251±46		427±43	Bx	711±96	A	***	***	*
18:1n-9	3508±1204		2906±529		6075±1335		5827±1998			***	
18:1n-7	271±6		283±51	y	489±118		506±23	x		***	
20:1n-9	87±44		29±8		35±0		32±7				
20:1n-7	192±33		192±37		250±14		206±31			*	
22:1n-11	378±272		150±12		147±11		93±33				
22:1n-9	165±122		80±29		70±7		54±14				
Σ monounsaturated	**5048±1356**		**3838±926**	**y**	**7668±1744**		**8549±422**	**x**		***	
18:2n-6	1446±443		1122±188	y	2360±520		2512±369	x		***	
18:3n-6	156±67		105±70		113±22		122±9				
20:2n-6	148±73		43±12		62±3		58±4		*		
20:3n-6	49±1	A	34±0	B	45±5		36±2		***		
20:4n-6	160±71		86±32		87±7		87±2				
22:4n-6	195±127		33±15		25±7		30±17			*	*
22:5n-6	96±59		44±7		43±3		49±13				
Σ n-6 PUFA	**2400±465**		**1415±262**	**y**	**2735±549**		**3042±165**	**x**		**	*
18:3n-3	884±161		584±113	y	1369±283		1441±426	x		***	
18:4n-3	138±56		92±14		150±36		152±19				
20:3n-3	139±62	Ax	50±25	B	48±4	y	47±8		**	**	*
20:4n-3	106±46		80±26		97±8		89±12				
20:5n-3	576±292		535±84		673±73		571±155				
22:5n-3	393±175		263±63		300±9		233±44				
22:6n-3	1101±294		1250±238		1224±31		1022±211				
Σ n-3 PUFA	**3701±1196**		**3259±146**		**3861±360**		**3554±186**				
[1]Σ PUFA	**6621±1846**		**4759±1122**		**6738±957**		**6585±394**				

†S refers broodstock selection, capital letters denote differences between selection groups fed with the same diet during the spawning period, D refers broodstock diet, lowercase letters denote differences among differentially fed broodstock groups during the spawning period with similar fads2 expression. DxS means interaction of these two parameters. *p<0.1, **p<0.05, ***p<0.01

1Total PUFA also includes 16:2, 16:3 and 16:4 fatty acids.

6.4 Discussion

FM and FO are two sources with supply limitations and cannot foster the steady growth of the aquaculture sector. Strategies to maximize the utilisation alternative ingredients to the FM and FO in aquaculture species can contribute the reduce for the dependency of the feed ingredients such as FM and fish oil. Moreover, the improvements towards the better feed utilisation will reduce the cost of production while reducing the waste production. Studies by (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017) showed, gilthead sea bream offspring's VM/VO utilisation at 4 and 16 months-old juveniles can be improved by using broodstock diet as a nutritional programming tool. However, there is still uncertainty about the types of the fatty acid profiles that triggers this improvement in the feed utilisation. Therefore, the characterisation of fatty acids involved in the nutritional programming is important to improve the n-3 LC-PUFA mechanism in the offspring. In addition, this study aims to contribute to this growing area of research by exploring the combined effects of selection and nutritional programming to further improve the utilisation of VM/VO diets at the on-growing stage of the gilthead sea bream.

In the present study, the broodstock diets are formulated by using the previous data obtained from (Izquierdo *et al.*, 2015)(Turkmen et al. submitted). In these studies, the researchers tested 4 different diets with increasing levels of FO substitution with VO (0, 60, 80 and 100% VO), hence, different levels of dietary 18C to LC-PUFA, found that 80% replacement lead reduction spawning quality parameters while 60% replacement performed in par with 100%FO diet. In the means of the replacement levels and its effects on the spawning quality, the results corroborate the findings of a great deal of the previous works but also shows that 70% of replacement had no effects on the spawning quality parameters in the broodstock groups within the same selection groups. It is important to bear in mind that the n-3 LC-PUFA contents of 70% replacement diet was comparable with the previous diets used by 60% percent of replacement of the study by (Izquierdo *et al.*, 2015). However, selection of the broodstock profoundly affected the spawning quality even within the groups either fed F or V diet during the spawning, LD groups showed significantly lower number of total eggs and 3dah larvae at the end of the trial. Besides, the same trend was observed for fertilised, viable and

hatched eggs although no statistical differences were found. As far as our knowledge, this is the first study regarding the effects of broodstock selection for *fad2* expression levels in gilthead sea bream. The importance of the *fads2* gene for the reproduction in mammals is clear as studied in *fads2* -/- mouse (Stoffel *et al.*, 2008). Researchers were observed that males were not able to produce mature sperm and folliculogenesis in females was disrupted in *fads2* -/- individuals. (Stoffel *et al.*, 2008). On the other hand, n–3 LC-PUFA level in broodstock diets have effects on the egg production and egg quality in various fish species including Gilthead sea bream (Fernández-Palacios *et al.*, 1995).

Nevertheless, it has been also observed in rodents, differences in the biochemical compositions such as lipid composition of maternal diets may lead differential offspring phenotypes by regulating gene expression patterns through the epigenetic mechanisms such as DNA methylation of the promoter (Hoile *et al.*, 2013). However, in the present study, to restrain this factor broodstock individuals fed with isoproteic, isoproteic and isoenergetic diets. Moreover, no differences in the biochemical analysis of the egg composition was observed in the differently fed broodstock groups thus, the differences observed in the progeny is mostly related to the fatty acid composition rather than the changes in the other biochemical parameters.

At the present study, larval growth was affected by the feeding different diet of broodstock selected for *fads2* expression levels. Despite the fact that larvae were kept in similar conditions and fed with the same diets, the growth were higher in FHD group in comparison with VHD group at 3 dah. On the other hand, the larvae obtained from the VHD group were able to catch up the growth of FHD at 15dah and 30dah. In mammals, as well as in fish, it has been shown in many studies that nutritional programming through parental diets in offspring weight, metabolism and immune responses depending on the dietary interventions during the early periods of the developments (Sinclair *et al.*, 2007; Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017; Engrola *et al.*, 2018)(Turkmen et al. submitted). Furthermore, the selection of broodstock high *fads2* expression groups HD was improved the FCR values of the of the juveniles at 6-months of age, indicating a better utilization if challenged with high VM and VO diets. This FCR improvements in the selected fish noticeably shows the selection with of the broodstock *fads2* gene

expression in broodfish improves the ability of fish to cope with high VM VO content in the juvenile's diet while nutritional programming through 18C fatty acid rich diet did not induce any beneficial effects on this parameter. The underlying mechanism of the feed utilization is not presented in this study since however, it has been well documented in the previous papers that, fatty acid composition changes in 18C fatty acid accompanied by a reduction of n-3 LC-PUFA in the broodstock diets led up-regulation of the some lipid related genes at larval (30 dah), early (4-months-old) and late juvenile stages (16-months-old) such as *fads2*, *elovl6, lpl* and *cpt1* (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017) in gilthead sea bream. Moreover, studies in Atlantic salmon showed reduction in both precursors and products availability led a nutritional programming effects at the juvenile stage (Clarkson *et al.*, 2017), which later-on explained more-in depth on the molecular mechanisms involved in the adaptation of the individuals (Vera *et al.*, 2017). The relationship between 18C precursors and DHA synthesis through the *de novo* biosynthesis is well demonstrated in rodent studies, the n-3 PUFA biosynthesis is very sensitive to the total PUFA and LA intakes (Gibson *et al.*, 2013). The relevance of PUFA in nutritional programming in gilthead sea bream is clearly supported by the current findings. Thus, this relation should be taken into account for the potential nutritional programming studies.

At the end of the trial, although the juveniles fish fed with the same diets, muscle fatty acid composition altered by the selection and broodstock feeding history by mainly increasing levels of saturated fatty acid in LD groups if fed with V diet during the spawning period due to increase in fatty acids 14:0 and 16:0 and 18:0.

Nevertheless, no differences were observed in muscle contents of EPA and DHA among the experimental groups or total n-3 LC-PUFA content. Thus, fillet fatty acid profiles regarding the EPA and DHA only beneficial roles of the selection was the differences in growth. The highest growth observed in FHD group showed around 40% more than the lowest growth group (VLD). The proportional growth of the muscle tissue is advantageous for both the producer and the consumer perspective.

The study contributes better understanding implementing novel strategies to maximize the utilization of VM and VO sources in the fish diets. In this study we aim to

assess the effects of the broodstock selection and the effects of 18C rich diets on broodstock performance, and possible nutritional programming effects on the progeny. In summary, this study shows, broodstock selection for higher fads2 expression markedly improved the reproduction success and offspring growth. On the other hand, abundancy in the precursors in the broodstock diets without a reduction in the n-3 LC-PUFA products did not have a positive nutritional programming effect. Thus, reduction of the n-3 LC-PUFA end products accompanied with 18C precursors seems to be required to nutritionally program the fish for better utilization of plant-based diets as demonstrated in the previous studies.

6.5 Acknowledgments

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The authors declare no conflicts of interest.

6.6 Author contributions

S.T. conducted all experiments, analyzed and evaluated all biological, biochemical, molecular analysis. H.X. helped the feeding of the fish of the juvenile experiment. Molecular biology samples were analysed by S.T. an H.X. with the supervision of M.J.Z. All trials were designed by S.T. D.M. and C.M.H., and M.I. supervised the entire work. L.R. was involved in the formulation and the preparation of the diets. The manuscript was written by S.T. and M.I.

Chapter 7

Broodstock selection and nutrition affect lipid metabolism related genes and methylation of the fatty acid desaturase 2 gene (*fads2*) promoter in gilthead sea bream offspring

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To be submitted…

Abstract:

Previous research has shown, polyunsaturated fatty acids (PUFA) in the early developmental stages plays a key role in regulating n-3 LC-PUFA metabolism of the offspring in mammals and other animals such as fish. However, it is not well understood if the regulation is driven by inadequate supplementation of the precursors (18:2n-6, LA 18:3n-3, ALA) and/or the products (20:4n-6, 20:5n-3, 22:6n-3) of n-3 long chain PUFA synthesis in the parental diets which modifies the available nutrients during the embryogenesis. Moreover, epigenetic mechanism such as promoter methylation of *fads2* gene, which codifies a rate-limiting enzyme in LC-PUFA not yet studied in detail in gilthead sea bream. In addition, it is not yet investigated the potential of selection of the parents using the *fads2* expression levels in peripheral blood cells as a biomarker in parents before the spawning period. To test these factors, present study consisted three phases, in the first phase broodstock were selected based on their *fads2* expression and in the second phase, each broodstock were fed with an ALA-rich diet to induce the nutritional programming, or a control diet. Lastly, in the third phase the progeny obtained from these groups were challenged with a challenge diet at juvenile stage. This study showed selection by *fads2* expression can improve utilisation of the low n-3 LC-PUFA diets of offspring, however, an ALA-rich diet during the embryogenesis cause negative effects on growth of the offspring. Even fishes were fed with same diet, epigenetic analysis showed, selection and type of the broodstock diet caused modifications on promoter methylation of *fads2*.

Keywords: DNA methylation, digital droplet PCR, modulation of lipid metabolism, nutritional programming, parental nutrition

7.1 Introduction

Aquaculture, the fastest growing food production sector, still has a great potential to grow, since it only occupies around 0.05% of the continental shelf (Council, 2015; FAO, 2018). Therefore, aquaculture has an important mission to provide sufficient high-quality food for an incessantly growing world population. However, among the several limitations that aquaculture must overcome, the limited production of capture-derived ingredients such as fishmeal (FM) and fish oil (FO) can markedly constrain future growth of aquaculture (Tacon and Metian, 2008; Turchini *et al.*, 2009; Kaushik and Troell, 2010). In fact, fisheries capture, including that for production of FM and FO, mainly remains steady over the last decade (FAO, 2018). Both FM and FO present a high bioavailability and adequate nutritional compositions that contribute to fulfill essential amino acids and fatty acids requirements of fish species (Turchini *et al.*, 2009; Hardy, 2010). Alternatively, oils and meals obtained from terrestrial crops such as vegetable meals (VM) and vegetable oils (VO) are currently used to replace FM and FO (Turchini *et al.*, 2009; Hardy, 2010). Despite the great achievements to reduce FM in the diets of marine fish species (Kaushik *et al.*, 2004; Izquierdo, 2005; Benedito-Palos *et al.*, 2007; Le Boucher *et al.*, 2011; Le Boucher *et al.*, 2013; Shepherd and Bachis, 2014; Torrecillas *et al.*, 2017), complete replacement of FO remains to be a major challenge. For instance, complete substitution of FO adversely affects immune system and stress and disease resistance (Montero and Izquierdo, 2010; Torrecillas *et al.*, 2017; Torrecillas *et al.*, 2017) and reduces the fillet content in long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA, includes twenty or more carbon atoms), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), negatively affecting the nutritional value of fish flesh for humans (Izquierdo *et al.*, 2005; Rosenlund *et al.*, 2010; Eroldoğan *et al.*, 2013; Yılmaz *et al.*, 2016; Torrecillas *et al.*, 2017).

Despite VO are rich in 18C fatty acids, such as linoleic (LA, 18:2n-6) and α-linolenic (ALA, 18:3n – 3) acids, which are precursors for the synthesis of ARA (20:4n-6), EPA and DHA (Tocher, 2015). In generally speaking, marine fish species there is a limited and insufficient activity of the enzymes involved this synthesis than the freshwater species (Castro *et al.*, 2016; Monroig *et al.*, 2018). Thus, maximising the capacity of fish

to utilise VO sources rich in 18C PUFA precursors would contribute to sustain optimal growth, welfare and fillet quality of cultured fish. Among the different elongation and desaturation enzymes involved in n-3 LC-PUFA synthesis, Δ-6-desaturase enzyme (delta6) encoded by *fads2* gene is a rate-limiting step in the biosynthetic pathway in gilthead sea bream (Vagner and Santigosa, 2011 Izquierdo et al., 2008). LC-PUFA synthesis also involves chain elongation catalysed by elongases (*Elovl*) with different substrate preferences (Monroig *et al.*, 2011). Among them, *Elovl6* is a key lipogenic enzyme that elongates long-chain saturated and monounsaturated fatty acids of 12, 14 and 16C, which has received much attention due to its relation to metabolic disorders (Matsuzaka and Shimano, 2009). In turn, LC-PUFA may have a direct effect on the expression of other genes related to lipid or carbohydrate metabolism (Clarke, 2001). Lipoprotein lipase (*lpl*) facilitates the tissue uptake of circulating fatty acids (Bell and Koppe, 2010) from lipoproteins and its expression in the liver can be regulated by n-3 PUFAs (Raclot *et al.*, 1997). The provision of energy is accomplished by β-oxidation of free fatty acids transported into the mitochondria in the form of fatty acyl-carnitine esters by carnitine acyltransferases, such as carnitine palmitoyltransferases (*cpt*) (Sargent *et al.*, 2002). Replacement of FO by VO changes the fatty acid composition of liver and muscle, affecting the β-oxidation capacity and regulating the expression of *cptI* and *cptII* genes (Kjaer *et al.*, 2008; Leaver *et al.*, 2008; Vestergren *et al.*, 2012; Xue *et al.*, 2015). β-oxidation also takes place in the peroxisome and is modulated by peroxisome proliferator activator receptors (PPARs). Three different PPAR isoforms (α,β,γ) have been characterised in gilthead sea bream, *ppar*□ being the major form expressed in the liver (Leaver *et al.*, 2008). PPARs are nuclear receptors that regulate differentiation, growth, and metabolism and in mammals, epigenetic mechanisms have been described to regulate these processes involving all the PPARs isoforms (Corbin, 2011). For instance, feeding pregnant rats a protein-restricted diet reduces methylation of the *ppar*□ promoter in the offspring, and the hypomethylation persists into adulthood (Lillycrop *et al.*, 2008). Finally, another gene potentially regulated by LC-PUFA is cyclooxygenase-2 (*cox2*), a key enzyme in prostanoid biosynthesis (Ishikawa and Herschman, 2007).

Recent evidences suggest the possibility of routing the offspring metabolic pathways by the inclusion of VO in broodstock diets, improving VM and VO utilisation

by the offspring in gilthead sea bream (Izquierdo *et al.*, 2015; Turkmen *et al.*, 2017). Therefore, increasing levels of substitution of FO by a VO up to 80% in broodstock diets up-regulate *fads2* expression in offspring larvae and improve the utilisation of low FM FO diets in the juvenile stage (Izquierdo *et al.*, 2015). This improved utilisation of low FM FO diets is persistent even in the 16-months-old offspring and affects the expression of some key genes encoding enzymes related to lipid utilisation and LC-PUFA biosynthesis including lipoprotein lipase *lpl*, *cpt1*, *elovl6* (Turkmen *et al.*, 2017). More recently, it has been demonstrated also that the selection of broodstock by their *fads2* expression levels in blood after one month feeding a low FO high linseed oil (LO) diet improves spawning performance as well as the growth of the offspring when nutritionally challenged with a low FM FO diets at 6-months-old (Turkmen *et. al.*, submitted). On the other hand, increase in dietary ALA without a proportional reduction in LC-PUFA led to an adverse nutritional programming effect on the offspring, resulting in lower growth than the offspring obtained from 100%FO fed broodstock. However, the effect of such ALA-rich broodstock diet or the selection of fish with a high *fads2* expression on offspring liver biochemical and fatty acid composition or the potential molecular mechanisms involved have not been yet studied.

Nutritional programming through parent diets has been well studied in humans, where early nutritional interventions during periods of the developmental plasticity may alter the risk of cardio-metabolic related diseases such as type 2 diabetes mellitus, hypertension, obesity, and osteoporosis (Burdge and Lillycrop, 2010). Moreover, LC-PUFA supplementation during early nutrition can lead to long-term effects on metabolism by affecting the epigenome through different epigenetic mechanisms including DNA methylation (Lillycrop and Burdge, 2018). Indeed, in murine models studies, maternal fat intake alters ARA and DHA contents in the liver, what was related to the epigenetic regulation of *Fads2* gene promoter and the expression of the same gene (Hoile *et al.*, 2013). In fish, epigenetic studies are a quiet new area of research (Panserat *et al.*, 2019). Previous studies in fish also showed there was a negative correlation between methylation status of the putative promotor region of *fads2* gene in a fish species Japanese sea bass (*Lateolabrax japonicus*) (Xu *et al.*, 2014). However, it remains undetermined if such changes can alter transcription and metabolic processes

(Lillycrop and Burdge, 2018). To our knowledge, in gilthead sea bream there are no data regarding the epigenetic mechanisms involved in the nutritional programming effect of broodstock diets. Fortunately, the publication of the whole genome for this species (Pauletto *et al.*, 2018) is opening new opportunities to understand the potential epigenetic mechanisms involved.

The objective of the present study was to investigate the effects of broodstock selection by their *fads2* expression levels and broodstock feeding a diet rich in VO on their juveniles offspring ability to utilize a low FM and low FO diet. Additionally, the potential changes in liver fatty acid composition, expression of specific genes involved in lipid metabolism and LC-PUFA biosynthesis, and the methylation status of the *fads2* gene promoter region were studied.

7.2 Materials and methods

All the mentioned experiments below were conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at Fundación Canaria Parque Científico Tecnológico (FCPCT), University of Las Palmas de Gran Canaria (Canary Islands, Spain).

7.2.1 Fish feeding

This research comprised three phases. In the first phase, broodstock were selected based on their *fads2* expression and in the second phase, each broodstock were fed with a VO diet to induce the nutritional programming, or a control diet. Lastly, in the third phase the progeny obtained from these groups were challenged with a diet low in FM and FO (5% FM - 3% FO) at juvenile stage (Figure 7-1).

In the first phase of the study, before the beginning of the spawning period, a total of 70 gilthead sea bream broodstock fish, including 42 females and 28 males, were fed a high VO and VM diet (Table 7-1) to trigger *fads2* expression and for the selection of broodstock individuals with high (*HD*) or low expression values (*LD*). Fish were fed at a 1% biomass ratio at 08:00 and 14:00 h daily, except Sundays, for 1 month. After this period, all fish were anaesthetized with 10 ppm clove oil/methanol (1:1 v/v) in seawater, and 2 mL blood samples were taken from the caudal vein with 2 mL sterile syringes

(Terumo Europe NV, Leuven, Belgium). Blood was collected in 2.0 mL K 3 EDTA tubes (L.P. Italiana, Milan, Italy), mixed and then, 1 mL of non-coagulated blood was transferred to 2 mL Eppendorf tubes. Blood samples were kept on ice during sampling and immediately centrifuged at 3000 rpm, 4ºC, for 10 minutes. Plasma was separated, and blood cells were snap frozen with liquid nitrogen and kept at -80 ºC until molecular studies were conducted. Broodstock showing the highest or the lowest *fads2* expression were separated into two groups, *HD* and *LD*, respectively.

In the second phase, to induce a nutritional programming in the offspring, 12 brood fish from the high- (*HD*) and 12 from the low- (*LD*) *fads2* expression group, with similar length and weight (Table 7-2), were distributed by couples into 12 experimental tanks in a flow-through system with filtered seawater (37.0±0.5‰ salinity, 19.59-21.30°C) at a renewal of 100% per hour and strong aeration. Tanks received indirect sunlight under a natural photoperiod between 10 and 12 h. Two experimental diets, diet F containing only FO and diet V containing both FO and VO (Table 7-3), were provided to each of the two *fads2* broodstock expression groups in triplicates, forming four experimental groups: F*HD*, fed with FO and showing high *fads2* expression; V*HD*, fed with VO and showing high *fads2* expression; F*LD*, fed with FO and showing low *fads2* expression; and V*LD*, fed with VO and showing low *fads2* expression. Both diets had similar levels of protein, lipid and ash content (Table 7-3).

Substitution of FO by LO in the broodstock diet V raised the content of 18C fatty acids such as LA and ALA, which are substrates for Fads2, with levels 1.8 and 18.1 times higher in V than in F diet, respectively. Conversely, 20 and 22C fatty acids, end products of fatty acid desaturation and elongation, such as ARA, EPA and DHA were similar (Table 7-2). Broodstock groups were fed twice a day (08:00 and 15:00 h) with a daily ration of diet corresponding to the 1% initial biomass of each experimental diet for 37 days. At the end of this feeding period, eggs were collected from all the groups and offspring were reared with the same commercial diets and feeding protocol until they reached 6-months of age (Turkmen *et al.*, submitted).

In the third phase, the offspring juveniles obtained from these 4 broodstock groups (F*HD*, F*LD*, V*HD* and V*LD*) were nutritionally challenged with a low FM and low

FO diet (5%FM and 3%FO), to determine the effect of broodstock selection and feeding on the offspring ability to use low FM and low FO diets. For that purpose, 6-months-old juvenile offspring from each broodstock group were distributed into twelve 500 L tanks and fed the challenge diet (Table 7-4) twice a day at 08:00 and 14:00h for 60 days, except Sundays, for 2 months.

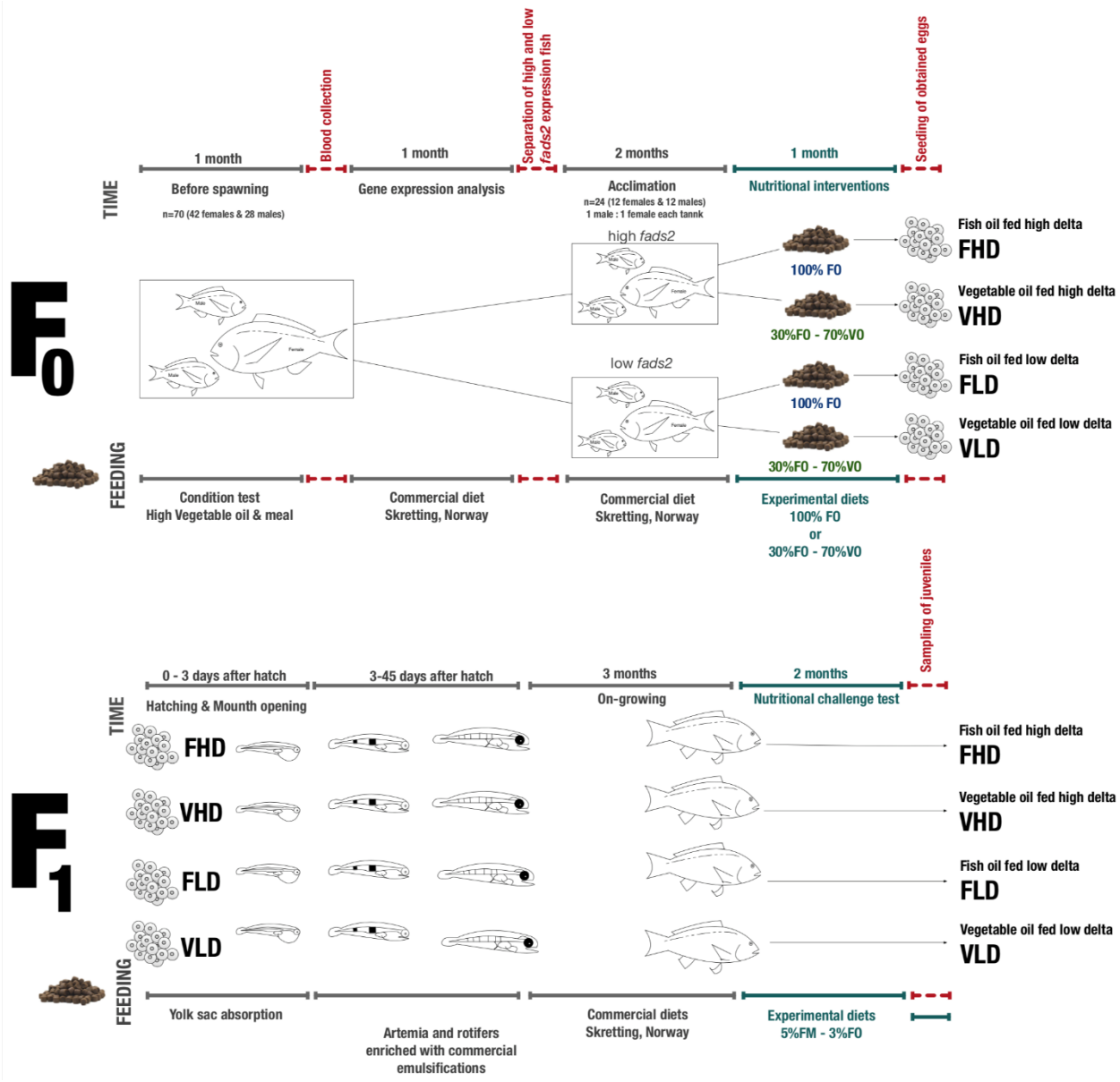

Figure 7-1 I Experimental design and sampling points, for the simplicity only one replicate shown in the figure, in the experiment all groups were tested in triplicates. Sampling points were shown as , and experimental periods when fish were fed with different diets are shown as .

Feed consumption was daily recorded. Growth was determined by measuring wet body weight after 24 h starvation. Prior to all measurements and samplings, all fish were

anaesthetized with 10 ppm clove oil/methanol (1:1 v/v) in seawater. Specific growth rate (SGR) values were calculated using: [Ln (final weight. g) – Ln (initial weight. g)] / (number of days) × 100.

7.2.2 Molecular studies

7.2.2.1 Broodstock selection

For each 200 µL of blood cells kept in 2 mL Eppendorf tubes, 1 mL of TRI Reagent (Sigma-Aldrich, Missouri, U.S.A.) was added.

Table 7-1 | Main ingredients*, energy, protein and % total fatty acids contents of diets used in conditioning test

Main Ingredients (%)	%	Proximate Composition	(% dry matter)
Fish meal SA[1] 68 super prime	5.00	Crude lipids	21.7
Fish meal alternative protein sources[2]	54.50	Crude protein	45.1
Rapeseed meal cake	11.30	Moisture	9.0
Wheat	6.89	Ash	5.4
Fish oil SA[1]	3.00		
Vegetable oil mix[3]	13.00	Gross Energy (MJ/kg, as is)	22.5
% total fatty acids		% total fatty acids	
14:0	6.6	18:3n-3	11.8
14:1n-5	0.1	18:4n-3	0.4
15:0	0.1	18:4n-1	0.0
16:0ISO	0.0	20:0	0.4
16:0	12.3	20:1n-9	0.0
16:1n-7	2.1	20:1n-7	1.0
16:1n-5	0.1	20:1n-5	0.1
16:2n-4	0.2	20:2n-9	0.0
17:0	0.3	20:2n-6	0.1
16:3n-4	0.1	20:3n-9	0.0
16:3n-3	0.0	20:3n-6	0.0
16:3n-1	0.0	20:4n-6	0.2
16:4n-3	0.4	20:3n-3	0.0
18:0	3.2	20:4n-3	0.1
18:1n-9	32.3	20:5n-3	2.5
18:1n-7	2.3	22:1n-11	0.1
18:1n-5	0.0	22:1n-9	0.3
18:2n-9	0.0	22:4n-6	0.0
18:2n-6	20.3	22:5n-6	0.1
18:2n-4	0.1	22:5n-3	0.3
18:3n-6	0.1	22:6n-3	1.7
18:3n-4	0.0		

* Please see Torrecillas *et al.*, (diet code 5FM/3FO for the complete list of feed ingredients.
1South American, Superprime (Feed Service, Bremen, Germany).
2Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
3Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

To each tube, four pieces of 1 mm diameter zirconium glass beads were added, and the sample homogenised in a TissueLyzer-II (Qiagen, Hilden, Germany) for 60 s at a frequency of 30/s. Homogenates were then diluted with 250 µL chloroform and centrifuged at 12000 G for 15 min at 4 °C. The clear upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to the membrane. After that, RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) and the protocol supplied by the manufacturer.

Table 7-2 | Formulation, main ingredients and biochemical composition of the nutritional programming diets supplied 1 month before egg collection for larval rearing

Raw material (%)	100% F0 (F)	30% F0-70% V0 (V)
Meals from marine sources[1]	50.0	50.0
Sunflower cake	13.2	13.2
Soya cake[2]	10.0	10.0
Fish oil[3]	8.0	2.4
Linseed oil[4]	-	5.6
Wheat	9.9	9.9
Corn gluten 60	7.0	7.0
Drying / wetting	0.9	0.9
Vitamin & mineral premix[5]	1.0	1.0
Vitamin E powder (50%)	0.1	0.1
Biochemical composition (% of dry matter)		
Moisture	9.1	8.8
Protein (crude)	56.3	56.1
Lipids (crude)	17.2	17.1
Ash	8.6	8.5
Energy - gross (MJ kg^{-1})	21.2	21.2
Fatty acids (% of total fatty acids)		
16:1n-7	7.1	4.3
18:2n-6	5.6	9.9
18:3n-3	0.9	16.3
20:1n-7	12.4	6.8
20:4n-6	0.4	0.3
20:5n-3	6.3	4.8
22:1n-11	15.7	8.6
22:6n-3	7.1	6.0

1 Contains Fish meal NA LT 70, Fish meal SA 68, Feed Service Bremen, Germany.
2 48 Hi Pro Solvent Extra. Svane Shipping, Denmark
3 South American fish oil, LDN Fish Oil, Denmark
4 Ch. Daudruy, France
5 Supplied the following vitamins (mg/kg): A 3.8, D 0.05, E 102.4, K3 9.8, B1 2.7, B2 8.3, B6 4.8, B12 0.25, B3 24.8, B5 17.2, folic acid 2.8, H 0.14, C 80; minerals (mg/kg): cobalt 0.94, iodine 0.7, selenium 0.2, iron 32.6. manganese 12, copper 3.2, zinc 67; other (g/kg): taurine 2.45, methionine 0.5, histidine 1.36, cholesterol 1.13. DSM, (Netherlands), Evonik Industries (Germany), Deutsche Lanolin Gesellschaft (Germany)

Real-time quantitative PCR was performed in an iQ5 Multi-colour Real-Time PCR detection system (Bio-Rad) using *actb* as the housekeeping gene in a final volume of 15

μL/reaction well, and with 100 ng of total RNA reverse-transcribed to complementary DNA (cDNA).

Housekeeping gene, cDNA templates and reaction blanks were analysed in duplicate. Primer efficiency was tested with serial dilutions (1:5, 1:10, 1:100 and 1:1000) of a cDNA pool. Sequences of the primers used in this study for Real-time quantitative PCR analysis of *fads2* expression were *actb* (GeneBank access no: X89920) 5'-3' (F): TCT GTC TGG ATC GGA GGC TC, 5'-3' (R): AAG CAT TTG CGG TGG ACG, *fads2* (GeneBank access no: AY055749) 5'-3' (F): CGA GAG CCA CAG CAG CAG GGA, 5'-3' (R): CGG CCT GCG CCT GAG CAG TT. Ninety-six-well PCR plate was used to analyse each gene, primer efficiency and blanks. Melting-curve analysis was performed to confirm the amplification of a single product after each run. Fold expression of each gene was determined by 2ˆ(-delta-delta CT) method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). PCR efficiencies were similar, and no efficiency correction was required (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

7.2.2.2 Digital droplet PCR analysis for absolute gene expression

Tissue RNA extraction was made using a similar protocol described above for the qPCR. Absolute gene expression analysis was performed with Droplet Digital PCR (ddPCR) using Bio-rad QX200 (Hercules, California, U.S.A.) systems, using the same cDNA obtained as above. Samples were prepared using the workflow provided by the manufacturer. In summary, for each gene, master mixes were prepared including 10 μL EvaGreen super mix (Bio-rad, Hercules, California, U.S.A.), 0.2 μL F primer, 0.2 μL R primer, 7.6 μL MilQ water and 2 μL cDNA (approx. 20 ng cDNA). Then, droplets were generated using droplet generator Bio-rad QX200 (Hercules, California, U.S.A.) and were transferred to 96 well microplates for PCR in a thermal cycler (Bio-rad C1000 Touch, Hercules, California, U.S.A.). Following PCR amplifications, droplets were read in a droplet reader (Bio-rad QX200, Hercules, California, U.S.A.) to determine absolute gene expression. Readings lower than 12000 droplets were not used for the gene expression.

7.2.2.3 DNA methylation analysis

DNA was extracted from liver samples using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, California, USA) following the manufacturer's instructions. Quantity and

quality of DNA were assessed by NanoDrop 2000c Spectrophotometer (Thermo Scientific, Massachusetts, USA) and integrity was analyzed in 2% agarose gels. Samples were stored at -20ºC until further processing. DNA was bisulfite converted using the EZ DNA Methylation Gold (Zymo Research, California, USA) bisulfite conversion kit following the manufacturer's recommendations. Primers were designed using the PyroMark assay design software (version 2.0.01.15) to hybridize CpG-free sites and to have the highest possible Tm. Reverse primers were labelled with biotin at the 5'-end to allow the capture of the biotinylated strand of the amplified DNA for further pyrosequencing. The target sequence was located within a previously identified CpG island at the *fads2* gene promoter, as determined by MethPrimer (http://www.urogene.org/methprimer/), and it contains 10 CpG sites. Search parameters for CpG islands were: length ≥ 200, C+G content ≥ 50%, ratio of observed /expected CpGs ≥ 0.60 and window size= 100. Bisulfite-converted DNA was amplified by PCR, using the Invitrogen™ Platinum™ Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA) and forward- and reverse- primers at 1 μM each in a total volume of 25 μL. Two regions were examined and were referred as 'left' and 'right' according to their relative position 5' to 3' within the identified CpG island. Each region was amplified by a forward (F) and a biotinylated reverse primer (R), and pyro-sequenced with a sequencing primer (Seq) as follows:

Left F: GTTGTAATTGAGGGAAAGTGTAGAA,

Left R: [btn]CACCCACTCATTCAATACAAATTC,

Left Seq: AGGGAAAGTGTAGAAG;

Right F: GTTGTAATTGAGGGAAAGTGTAGAAG,

Right R: [btn]TCATTCACCCACTCATTCAATACAAATTC,

Right Seq: GGTGGTTTAGGATATATTG.

Reactions were performed in an Eppendorf Mastercycler Ep Realplex (Eppendorf, Hamburg, Germany) with the following cycling: 95ºC for 5min, followed by 35 cycles of 95ºC for 45 s, 60ºC for 45 s, and 72ºC for 1.5 min and a final extension at 72ºC for 5min. PCR products were checked by 1% agarose gels to ensure specificity before pyrosequencing. Pyrosequencing reactions were carried out using the PyroMark Q24

instrument (Qiagen, Hilden, Germany) and data analysis was done using the PyroMark Q24 software. Only pyrosequencing reactions that passed the quality test were included in the analysis. Methylation values were expressed per position as the average of replicates, and as average methylation for the fragment as the mean of values across all sites analyzed. Sample size in the pyrosequencing assay for the different groups were F*HD* (n=4), F*LD* (n=5), V*HD* (n=3), V*LD* (n=5). Controls to assess pyrosequencing quality were included in each run.

7.2.3 Statistical analysis

The results were expressed as mean ± standard deviation (n=3) if not otherwise stated in material methods section, tables and figures. Data were compared statistically using analysis of variance (ANOVA) at a significant level of 5%. All variables were checked for normality and homogeneity of variance using the Kolmogorov–Smirnoff and the Levene tests, respectively (Sokal and Rohlf, 1969). If significant differences were detected with ANOVA, means were compared by the Tukey test. All data were analysed using IBM SPSS v23.0.0.2 for Mac (IBM SPSS Inc. Chicago, IL, U.S.A.).

Table 7-3 | Weight, length and *fads2* expression levels of the broodstock groups used in the present study

Groups			FHD	FLD	VHD	VLD
Weight	(kg)	♀	2.40±0.33	1.99±0.28	2.61±1.19	1.84±0.61
		♂	2.05±0.89	1.68±0.13	1.30±0.45	1.65±0.48
Lenght	(cm)	♀	45.75±0.35	42.83±1.44	45.33±5.50	41.83±3.75
		♂	45.50±1.41	39.83±1.15	36.66±2.02	40.83±5.05
fads2 expression*	(fold change)	♀	5.77±2.41^{a}	0.17±0.18^{b}	5.29±1.91^{a}	0.35±0.08^{b}
		♂	3.05±1.11^{a}	0.26±0.06^{b}	6.06±6.26^{a}	0.72±0.13^{b}

* Values in the same row have different letter indicates differences between groups (n=3, P<0.05). In case of similar values, no indications were made (P>0.05).

7.3 Results

After one month of feeding the high VO and VM diet (Table 7-1), there were differences in the *fads2* expression in broodstock fish, which was up to 23 times higher in females and 13 times higher in males than the lowest *fads2* expression of each sex (Table 7-3). Besides, after dividing the broodstock fish by their *fads2* expression, the resultant high *fads2* expression group showed significantly higher expression than the

low *fads2* group (P<0.05) (Table 7-3). No relation was found between fish weight and *fads2* expression of brood fish (R=0.0061).

The nutritional challenge of the juveniles with the very low FM and FO diet (5% FM and 3%FO) (Table 7-4) resulted in higher SGR values for offspring from broodstock with a higher *fads2* expression (F*HD* and V*HD*), than for those coming from parents with a lower expression (F*LD* and V*LD*) (P<0.001, 2-way ANOVA, Table 7-5), particularly, when parents were fed F diet (P<0.05, Figure 7-2). Besides, offspring from broodstock fed the V diet showed lower growth than offspring from parents fed the F diet (P<0.001, 2-way ANOVA, Figure 7-2). In offspring obtained from broodstock fed V diet (V*HD* and V*LD*), those from broodstock with lower *fads2* expression (V*LD*) showed significantly higher HSI (P<0.05, Table 7-5). Besides, in offspring from broodstock with lower *fads2* expression (F*LD* and V*LD*), those fed V diets (V*LD*) had higher HSI (P<0.05, Table 7-5). Thus, HSI was increased in offspring of parents with lower *fads2* expression (P<0.05, 2-way ANOVA), particularly when broodstock were fed the V diet, showing an interaction between selection and broodstock diets (P<0.05, Table 7-5).

Crude protein, crude lipid and ash contents of liver were not significantly different among the experimental groups (P>0.05, Table 7-5), despite a 6-13% increased hepatic lipid content in juveniles from broodstock with lower *fads2* expression. However, offspring from broodstock with lower *fads2* expression (F*LD* and V*LD*) showed significantly higher monounsaturated fatty acids 16:1n-5 and 20:1n-5, as well as some mid-chain PUFA such as 16:3n-4, 18:2n-4 and, particularly, 18:2n-6 (substrate for Fads2), or the LC-PUFAs 20:2n-9, 20:3n-6, 20:5n-3, 22:5n-3 or 22:6n-3 (P<0.05, Table 7-6).

The comparison of juveniles from broodstock fed the V diet (V*HD* and V*LD*) showed that in V*LD* juveniles, products of Fads2 activity such as 20:2n-9 (P<0.05) and 20:3n-6 (P<0.1) were reduced, or did not change, such as 18:2n-9, 18:3n-6, 18:4n-3 or 20:4n-3 (P>0.05), and Fads2 substrates such as 20:1n-9, 20:2n-6, 18:1n-9, 18:2n-6, 18:3n-3 or 20:3n-3 did not significantly change either. Besides, F*LD* and V*LD* fish showed a reduction in the substrates for Elovl6*,* 16:0 (P=0.06), but 18:1 products was similar among the experimental groups (P>0.05) (Table 7-6). Broodstock diet had no

significant (P>0.05) effect on fatty acid profiles of the liver tissue of the offspring juveniles for the analysed fatty acids (Table 7-6), except for a tendency to lower values of saturated fatty acids (Table 7-6, P>0.05). No interaction of broodstock diet and *fads2* expression was observed on fatty acid profiles, except for the content of a minor fatty acid, 16:0ISO (P<0.05, Table 7-6).

Regarding expression of selected genes, juveniles from parents with low *fads2* expression (F*LD* and V*LD*) showed a significantly (two-way ANOVA, P<0.001, Figure 7-3) higher expression of *elovl6,* particularly when broodstock were fed with diet F. Thus, *elovl6* expression was approximately 2 times higher in F*LD* group if compared with F*HD* (P<0.05) (Figure 7-3), whereas between juveniles from parents fed V diet, the expression of this gene was only 1.3 times higher in V*LD* fish than in V*HD* group (P<0.05) (Figure 7-3). Indeed, feeding broodstock with the V diet significantly (two-way ANOVA, P<0.01, Figure 7-3) not up-regulated *elovl6* expression in the offspring juveniles, particularly in those from broodstock with lower *fads2* expression. Thus, juveniles from lower *fads2* expression broodstock showed a significantly lower expression in *elovl6* when their parents were fed V diet (V*LD* juveniles) than when they parents were fed F diet (F*LD* juveniles, P<0.05) (Figure 7-3). Therefore, there was a significant interaction between broodstock *fads2* expression and broodstock diet on the expression of *elovl6* in their juveniles (two-way ANOVA, P<0.01, Figure 7-3). The expression of *cpt1* followed a similar trend, although the down-regulation effect of broodstock diet V had a lower significance (two-way ANOVA, P<0.05, Figure 7-3). Thus, juveniles from parents with low *fads2* expression (F*LD* and V*LD*) showed a significantly (two-way ANOVA, P<0.001, Figure 7-3) higher expression of *cpt1* than those from parents with higher expression (F*HD* and V*HD*). Despite both selection of broodstock for low *fads2* expression and feeding diet V tended to down-regulate juveniles expression of *fads2* and *cox2*, there were no significant differences among juveniles in the expression of this two genes (P>0.05, Figure 7-3). There was neither differences in *lpl* nor *ppara* gene expression (P>0.05, Figure 7-3). In general, a low level of cytosine methylation was found for the studied fragment of the *fads2* promoter. Methylation level was always <10% for individual CpG positions and <4% for the average of all CpG positions examined (Figure 7-4). However, few differences were noticed at individuals CpGs; methylation at

positions CpG2 and CpG3 in the offspring juveniles coming from the broodstock fed V diet was significantly higher when broodstock had a low *fads2* expression (V*LD*) than when they had a high *fads2* expression (V*HD*) (P<0.05) (Figure 7-4).

Table 7-4 I Formulation, main ingredients and biochemical composition of the nutritional challenge diets

Main Ingredients (%)	%	Proximate Composition	(% dry matter)
Fish meal SA[1] 68 super prime	5.00	Crude lipids	21.8
Fish meal alternative protein sources[2]	54.50	Crude protein	57.2
Rapeseed meal cake	11.30	Moisture	6.5
Wheat	6.89	Ash	6.7
Fish oil SA[1]	3.00		
Vegetable oil mix[3]	13.00	Gross Energy (MJ/kg, as is)	22.5
% total fatty acids		% total fatty acids	
14:0	4.69	18:3n-4	0.04
14:1n-5	0.17	18:3n-3	14.86
15:0	0.28	18:4n-3	0.91
16:0	13.67	20:0	0.21
16:1n-7	4.50	20:1n-9	0.37
16:1n-5	0.09	20:1n-7	7.25
16:2n-6	0.25	20:1n-5	0.37
17:0	0.13	20:2n-6	0.16
16:3n-4	0.14	20:4n-6	0.31
16:3n-3	0.14	20:3n-3	0.09
16:3n-1	0.04	20:4n-3	0.19
16:4n-3	0.22	20:5n-3	3.81
18:0	3.23	22:1n-11	9.35
18:1n-9	15.75	22:1n-9	1.00
18:1n-7	2.46	22:4n-6	0.04
18:1n-5	0.23	22:5n-6	0.03
18:2n-6	9.84	22:5n-3	0.28
18:2n-4	0.05	22:6n-3	4.49
18:3n-6	0.08		

Please see (Torrecillas *et al.*, 2017) (diet code 5FM/3FO for the complete list of feed ingredients.
1 South American, Superprime (Feed Service, Bremen, Germany).
2 Blood meal spray (Daka, Denmark), soya protein concentrates 60% (Svane Shipping, Denmark), corn gluten 60 (Cargill, Netherlands), wheat gluten (Cargill, Netherlands).
3 Linseed (2.6%) (Ch. Daudruy, France), rapeseed (5.2%) (Emmelev, Denmark) and palm oils (5.2%) (Cargill, Netherlands).

Although not statistically significant, the same trend was observed for all other positions analysed and consequently, the average value for V*LD* juveniles was higher than for V*HD* juveniles (P<0.05) (Figure 7-4). Two-way ANOVA of methylation data for CpG2 and CpG3 revealed that in any case the parents diet had a significant effect (CpG2: F=0.0006, P=0.981; CpG3: F=3.30, P=0.099). The level of *fads2* gene expression of parents (i.e., selection) had no effect on the methylation level at CpG2 (F=4.34, P=0.058)

while it had a significant effect at CpG3 (F=14.13, P=0.004). For both CpG positions, a significant interaction between parents' selection and nutrition was found for methylation level (CpG2: F=10.11, P=0.007; CpG3: F=5.75, P=0.037).

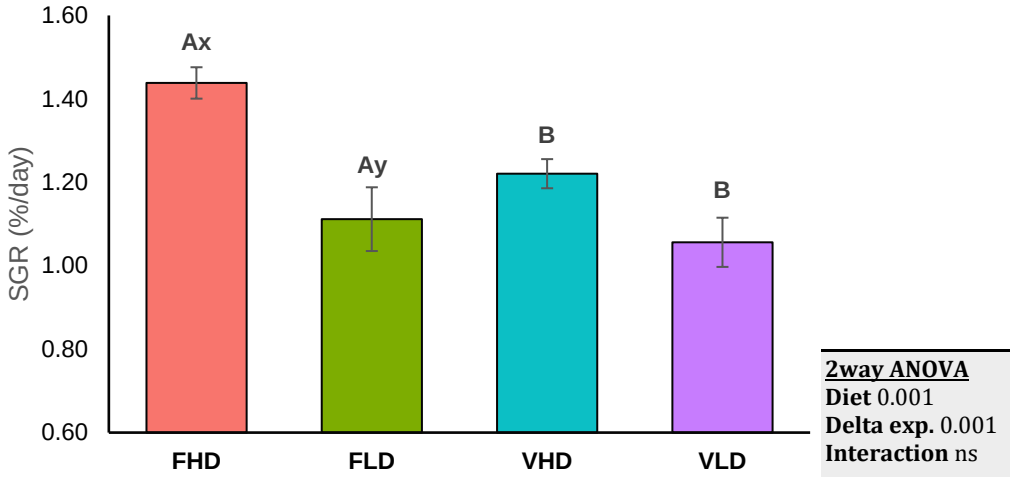

Figure 7-2 I Specific growth rate (SGR, %/day) of progeny obtained from different broodstock groups after nutritional challenge test with very low fishmeal and fish oil diets.

*Capital letters denotes differences between the same selection group (high fads2 or low fads2 groups) fed with different diets (F or V), lowercase letters show differences between the same diet groups (F or V) selected broodstock (high or low fads2) groups.
Specific growth rate (SGR. %/day) = [Ln (final weight. g) – Ln (initial weight. g)] / (number of days) × 100.

Table 7-5 I Juvenile liver biochemical composition and hepatosomatic index after the nutritional challenge test with a low FM/FO diet for 2 months

	Groups				Two-way ANOVA*		
Composition (%)	F*HD*	F*LD*	V*HD*	V*LD*	Broodstock Diet (D)	Selection (S)	D× S
Moisture	64.2±2.0	66.6±5.9	66.5±0.1	66.6±4.2	n.s.	n.s.	n.s.
Protein	11.1±0.3	10.1±2.4	12.1±0.4	11.2±1.3	n.s.	n.s.	n.s.
Lipids	13.9±2.1	14.1±4.9	12.1±1.8	11.0±2.1	n.s.	n.s.	n.s.
Ash	2.5±0.5	2.7±1.2	2.9±0.3	2.9±0.4	n.s.	n.s.	n.s.
HSI†	1.4±0.2	1.2±0.2^{B}	1.3±0.1^{b}	1.6±0.1^{Aa}	n.s.	0.05	0.03

*Values in the same row have different letter indicates differences between groups (n=3, P<0.05). In case of similar values, no indications were made (P>0.05).
† Hepatosomatic index (HSI) = (liver weight/fish weight) x 100

7.4 Discussion

Before the spawning season, and after one month of feeding broodstock with a high VM and VO diet to select them for their *fads2* expression, a wide variation in the *fads2* expression in peripheral blood cells was found. For instance, some females showed *fads2* expression values that were 23 times higher than the lowest values found in other females and, in males, highest values were up to 13 times higher than the lowest values for each sex. This variation, and the higher expression in female individuals, could be related to a potential higher requirement for DHA in females than in males, since this fatty acid plays essential roles in embryo development (Izquierdo *et al.*, 2001). In agreement with this hypothesis, female mammals maintain higher levels of DHA in liver and plasma phospholipids, in relation to their higher ability to synthesise DHA than their male counterparts (Burdge and Calder, 2006). Moreover, studies in humans showed a very high correlation between reproductive hormones, such as progesterone and estradiol, and *FADS2* expression or DHA concentrations (Childs *et al.*, 2012). Up to date, the knowledge about the relationship between reproductive hormones and *fads2* gene expression in fish is scarce. Further studies are being conducted to clarify this relationship in gilthead sea bream.

During the spawning season, eggs fatty acids composition reflected the diets profiles in the present study (Turkmen *et al.*, submitted) and in agreement to previous studies with the same species (Izquierdo *et al.*, 2001; Fernández-Palacios, 2005; Izquierdo *et al.*, 2015). After one month of feeding the low FM and low FO diet (5% FM

Table 7-6 l Fatty acid composition of livers after challenge test diets with substitution of FO by LO (% total fatty acids, mean ± SD, n = 3 (one pool of 5 livers per tank * 3)

	FHD	FLD	VHD	VLD	Diet (D)	Selection(S)	D*S
14:0	1.58±0.09	1.66±0.40	1.76±0.27	1.58±0.28	0.76	0.76	0.47
14:1n-7	0.01±0.00	0.01±0.00	0.02±0.00	0.02±0.00	0.14	0.69	0.52
14:1n-5	0.02±0.01	0.04±0.01	0.03±0.00	0.03±0.00	0.80	0.20	0.16
15:0	0.13±0.01	0.17±0.04	0.14±0.02	0.14±0.00	0.68	0.12	0.20
15:1n-5	0.02±0.00	0.02±0.00	0.02±0.01	0.02±0.00	0.88	0.51	0.11
16:0ISO	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.49	0.43	**0.03**
16:0	15.70±0.42	14.30±0.96	16.02±1.68	13.92±1.92	0.97	0.06	0.67
16:1n-7	2.26±0.17	2.49±0.44	2.57±0.23	2.43±0.23	0.48	0.79	0.31
16:1n-5	0.11±0.00	0.13±0.01	0.12±0.00	0.14±0.02	0.24	**0.04**	0.38
16:2n-4	0.05±0.02	0.09±0.05	0.07±0.02	0.08±0.01	0.71	0.11	0.37
17:0	0.07±0.01	0.11±0.04	0.09±0.02	0.10±0.01	0.52	0.09	0.27
16:3n-4	0.19±0.01	0.21±0.00	0.19±0.00	0.20±0.00	0.80	**0.00**	0.07
16:3n-3	0.03±0.00	0.04±0.01	0.04±0.00	0.04±0.01	0.67	0.08	0.08
16:3n-1	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.11	0.50	0.19
16:4n-3	0.03±0.01	0.05±0.04	0.04±0.02	0.05±0.01	0.63	0.17	0.46
18:0	6.16±0.54	5.38±0.17	5.86±0.37	5.40±0.49	0.57	**0.03**	0.52
18:1n-9	42.01±0.34	40.03±2.18	40.50±0.48	40.05±0.88	0.32	0.12	0.30
18:1n-7	2.52±0.05	2.60±0.14	2.54±0.07	2.56±0.04	0.86	0.31	0.54
18:1n-5	0.06±0.01	0.08±0.00	0.06±0.03	0.07±0.00	0.31	0.17	0.84
18:2n-9	2.04±0.10	2.29±0.49	2.26±0.33	2.69±0.88	0.35	0.30	0.78
18:2n-6	11.09±0.41	11.85±0.33	10.64±0.56	11.67±0.63	0.31	**0.01**	0.66
18:2n-4	0.06±0.01	0.09±0.02	0.07±0.00	0.08±0.01	0.85	**0.01**	0.26
18:3n-6	1.69±0.04	2.17±0.39	1.89±0.46	2.49±0.85	0.41	0.11	0.85
18:3n-4	0.06±0.00	0.07±0.01	0.07±0.01	0.07±0.00	0.32	0.20	0.11
18:3n-3	5.30±0.24	5.67±0.44	5.15±0.57	5.74±0.45	0.89	0.10	0.68
18:4n-3	1.21±0.01	1.60±0.23	1.41±0.39	1.80±0.53	0.36	0.09	1.00
18:4n-1	0.02±0.00	0.04±0.01	0.03±0.00	0.04±0.00	0.32	0.10	0.19
20:0	0.16±0.00	0.15±0.03	0.16±0.02	0.14±0.02	0.69	0.41	0.87
20:1n-9	0.12±0.01	0.14±0.02	0.12±0.01	0.13±0.01	0.51	0.19	0.69
20:1n-7	0.73±0.05	0.71±0.18	0.74±0.11	0.66±0.09	0.83	0.48	0.69
20:1n-5	0.07±0.00	0.08±0.01	0.07±0.01	0.08±0.00	0.49	0.04	0.45
20:2n-9	0.75±0.12	0.51±0.12	0.88±0.02	0.47±0.14	0.52	**0.00**	0.23
20:2n-6	0.26±0.02	0.25±0.02	0.24±0.01	0.23±0.02	0.11	0.23	0.92
20:3n-9	0.00±0.00	0.01±0.00	0.00±0.00	0.01±0.00	0.71	0.12	0.48
20:3n-6	0.27±0.03	0.22±0.03	0.31±0.02	0.21±0.04	0.37	**0.00**	0.19
20:4n-6	0.35±0.00	0.39±0.05	0.35±0.02	0.37±0.02	0.57	0.17	0.50
20:3n-3	0.23±0.02	0.22±0.01	0.21±0.01	0.21±0.01	0.07	0.46	0.67
20:4n-3	0.30±0.03	0.32±0.04	0.38±0.04	0.33±0.01	0.07	0.39	0.12
20:5n-3	0.96±0.12	1.42±0.38	1.21±0.10	1.42±0.16	0.37	**0.03**	0.36
22:1n-11	0.17±0.04	0.24±0.12	0.21±0.06	0.22±0.02	0.87	0.35	0.53
22:1n-9	0.27±0.01	0.26±0.04	0.26±0.04	0.24±0.02	0.37	0.35	0.57
22:4n-6	0.04±0.00	0.05±0.01	0.05±0.01	0.05±0.00	0.83	0.15	0.36
22:5n-6	0.10±0.00	0.12±0.01	0.10±0.01	0.11±0.01	0.90	0.05	0.13
22:5n-3	0.39±0.05	0.61±0.12	0.49±0.04	0.61±0.09	0.35	**0.01**	0.37
22:6n-3	2.37±0.16	3.09±0.51	2.60±0.18	3.10±0.38	0.56	**0.01**	0.57
Σ							
Saturated	23.80±0.84	21.77±1.59	23.03±1.78	21.29±2.58	0.57	0.11	0.89
16:1n	2.37±0.17	2.62±0.45	2.60±0.24	2.57±0.21	0.53	0.60	0.41
18:1n	44.59±0.32	42.70±2.04	43.53±0.93	42.68±0.91	0.46	0.09	0.48
20:1n	0.92±0.06	0.93±0.21	0.91±0.12	0.87±0.08	0.68	0.79	0.76
18:1/16:1	18.90±1.52	16.70±3.55	16.82±1.72	16.70±1.31	0.87	0.65	0.67

and 3% FO), offspring juveniles obtained from broodstock with high *fads2* expression showed better growth and lower liver contents of 18:2n-6, a substrate for Fads2, than juveniles from broodstock with low *fads2* expression. Interestingly, juveniles obtained from broodstock with high *fads2* expression showed the largest variation in the *fads2* expression in liver, as well as the highest values. However, there were no significant differences among fish groups in *fads2* gene expression, probably due to large variation among individual data. In the present study, the combined genetic and nutritional effects of the parents on the offspring growth resulted in higher growth in fish obtained from high *fads2* expression and F fed parents, and the growth was higher in high *fads2* than low fads2 groups regardless of the parental diet. Studies in humans showed that LC-PUFA metabolism in babies could be affected by the maternal *FADS2* genetic and epigenetic status (Lupu *et al.*, 2015). However, in the absence of a study investigating the heritability of *fads2* gene, it is difficult to interpret the reason behind these individual differences. However, our preliminary results showed high correlation between *fasd2* expression in broodstock and its progeny, if the broodstock is fed with high VM VO diets prior to gene expression analysis in the blood cells (Turkmen *et. al.*, in prep.). On-going studies with kinship-know brood fish will reveal more detailed information on the genetic variability and the parental effects of the selection based on *fads2* gene expression. Regarding putative epigenetic effects, results are in agreement with previous studies in gilthead sea bream showing the long-term effects of broodstock feeding with FO substitution by LO and a reminder diet at the early juvenile stage. This may also explain part of the variation observed in *fads2* expression in the progeny originated from high *fads2* expression parents (Turkmen *et al.*, 2017). Nevertheless, it should be taken into account that although the substitution levels of FO by LO were not so different between studies (60% in previous whereas 70% in the present study), Fads2 products (ARA, EPA and DHA) were higher in the present study in V diets, which may lead to lower variation in the V diet group.

In contrast, leading to a reduced growth in offspring juveniles fed the low FM and low FO diet, selection of broodstock with low *fads2* expression up-regulated *elovl6* expression in the offspring, in agreement with lower 16:0 in the liver of juveniles, respect to those from broodstock with high *fads2* expression. Elovl6 is a key rate-limiting enzyme

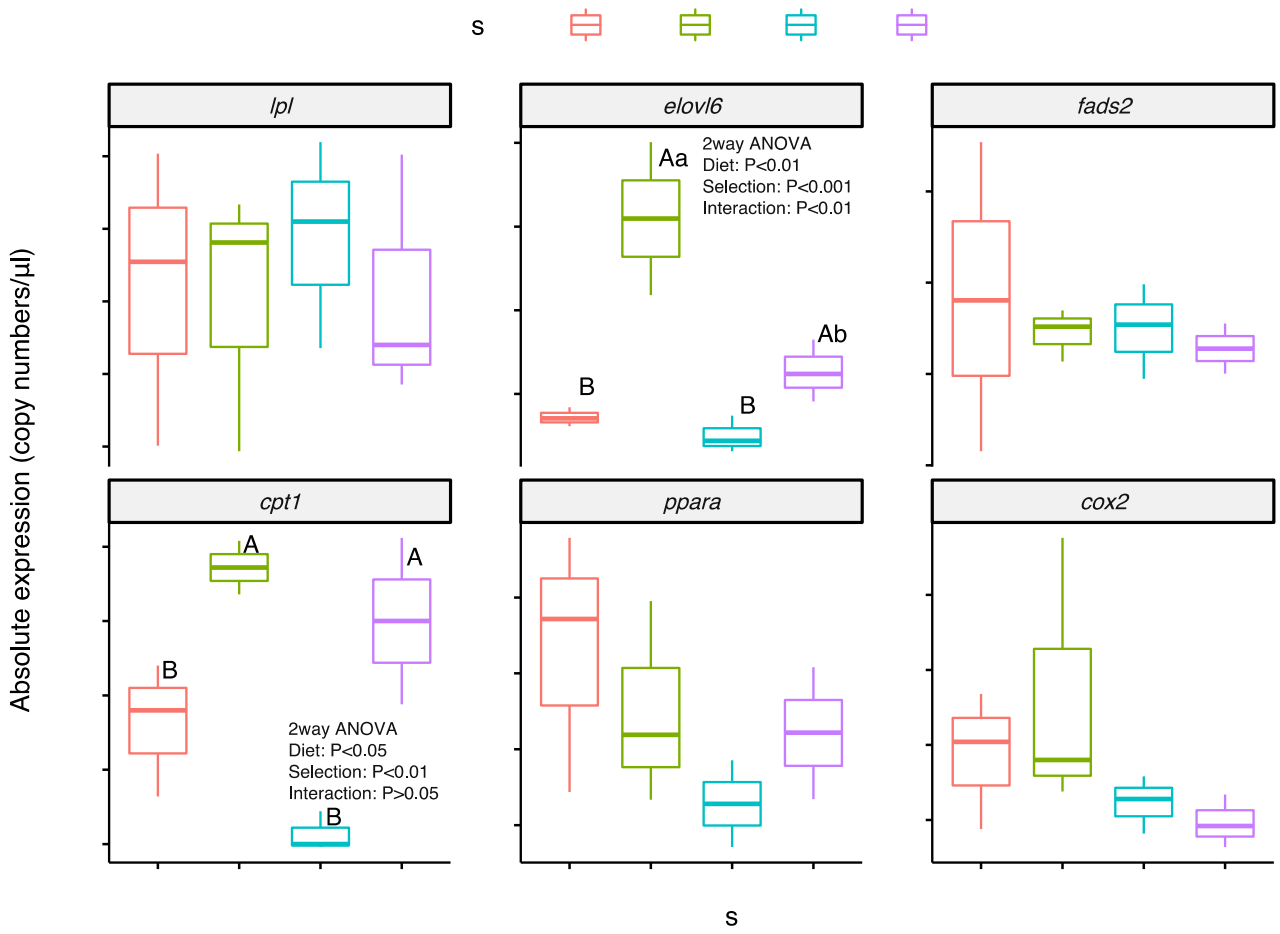

Figure 7-3 I Box-and-whisker plots of absolute gene expression (copy numbers/µL) of six different genes after challenging 6-months-old gilthead seabream individuals with high vegetable oil and meal feeds for 2 months.

Complete gene names; *lpl*: Lipoprotein lipase, *ppara*: Peroxisome proliferator-activated receptor alpha, *elovl6*: Elongation of very long chain fatty acids protein 6, *fads2*: Fatty acid desaturase 2, *cox2*: Cyclooxygenase-2, *cpt1*: Carnitine palmitoyltransferase I.

Indications are as follows: P values of two-way ANOVA analysis were shown inside the tables if P values were < 0.05. Letters indicate differences between groups, ns means no significance difference (P> 0.05). n=3 for all groups and genes..

in the long-chain fatty acid elongation cycle and it is involved in elongation of 16:0 and 16:1 to 18:0 and 18:1 fatty acid. Moreover, it has been shown that it is the only enzyme able to elongate 16:0 as it was shown in ELOVL-/- mice (Moon *et al.*, 2014). Nevertheless, 16:0 decreased in fish obtained from low *fads2* groups showed and 16:1 product in the livers showed a similar tendency, in line with the *elovl6* expression levels in the present study. However, the 18:1 fatty acid showed no clear trend in relation with

the *elovl6* expression, and this could be related to the relatively low levels of 18:0 (3.53%) and the high levels of 18:1n-9 (15.25%) fatty acids in the juvenile challenge diets.

Besides, *cpt1* was also in juveniles coming from low *fads2* groups were up-regulated, in comparison to those from broodstock with high *fads2* expression. Indeed, Cpt1 is a key enzyme for energy production through the β-oxidation of fatty acids transported into the mitochondria in the form of fatty acyl-carnitine esters by carnitine acyltransferases in fish (Sargent *et al.*, 2002). Therefore, the up-regulation of *cpt1* gene would imply an increase in liver β-oxidation, in agreement with a 6-13% reduction in hepatic lipid content of juveniles obtained low *fads2* groups although fat content is also influenced by the rate of lipid secretion by the liver. Down-regulation of both *elovl6* and *cpt1* in these groups were probably related, since, *Cpt1* gene expression in the liver of *Elovl6* knock-out rats is also down-regulated, leading to changes in the fatty acid ratios in liver (Matsuzaka and Shimano, 2009) similar to those described in this study. In addition, in the liver of *Elovl6* knock-out rats, changes in fatty acid chain length (decrease in LC-PUFA of more than 18C) and the ratio of fatty acids (C18:0/C16:0, C16:1/C16:0) reduced sterol regulatory element binding protein 1 (*Srebp-1*) and *Ppara* (Matsuzaka and Shimano, 2009). It has been also observed that PPARa has a very important role in energy metabolism, and its deficiency was found to be related to obesity in rats (Matsuzaka *et al.*, 2007). Thus, modulation of *elovl6* expression may be one part of the answer to the improved growth in high *fads2* selected fish. Moreover, when juveniles were obtained from broodstock fed the V diet, selection of broodstock with low *fads2* expression lead to the lowest growth and also to increased HSI in comparison to juvenile from broodstock with high *fads2* expression. Besides, juveniles from broodstock with low *fads2* expression showed significantly increased methylation at certain CpG positions within a CG island in the promotor region of *fads2*, particularly in positions CpG2 and CpG3. These results suggest a reduced ability of these juveniles to transcribe *fads2* in comparison to those from broodstock with high *fads2* expression and fed the V diet, which deserves further investigation.

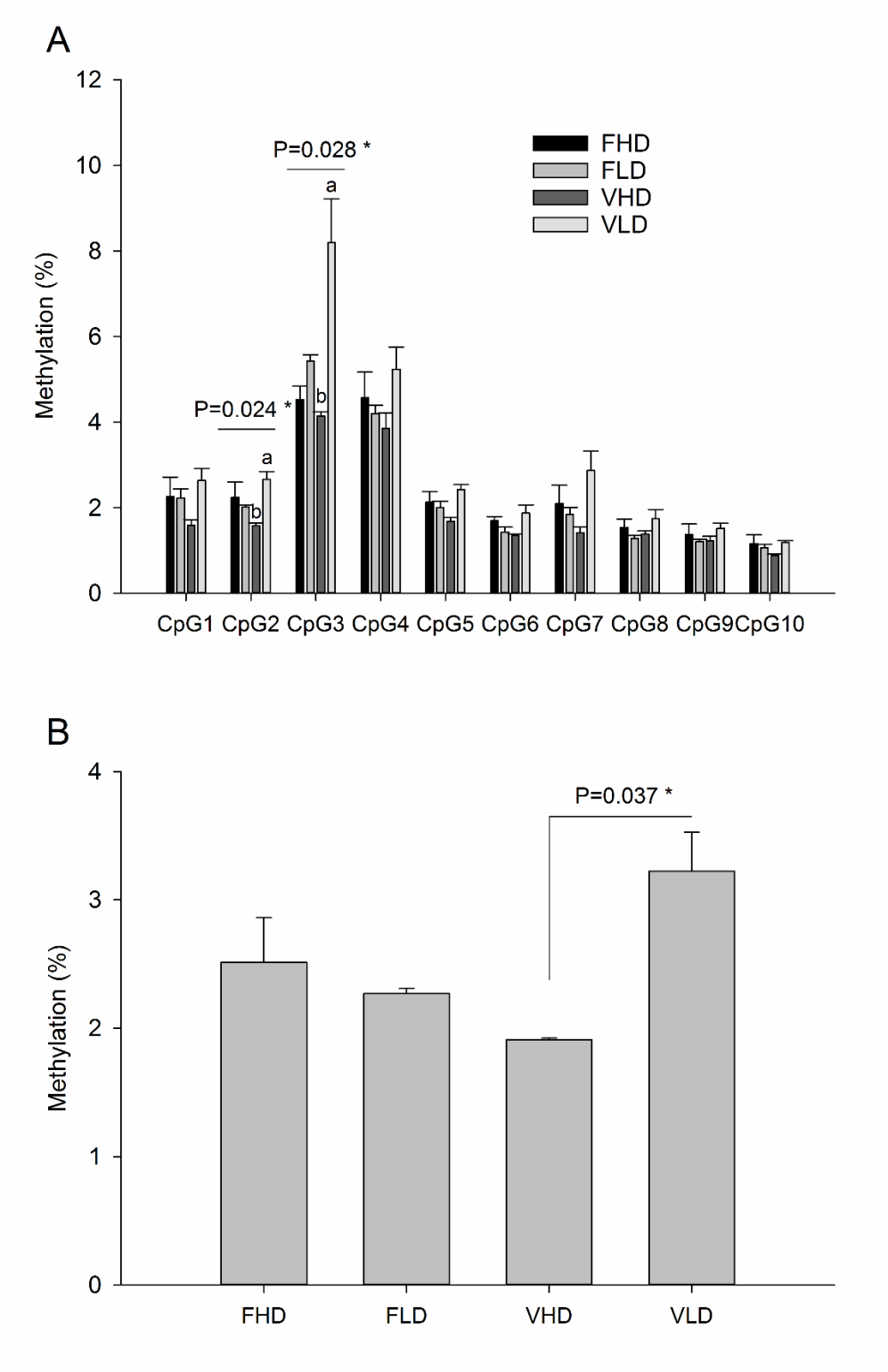

Figure 7-4 | A. Site-specific methylation level of 10 CpG positions in promoter region of the *fads2* gene, in livers of gilthead sea bream offspring juvenile obtained from selected broodstock fed with either 100% fish oil (F) or 30% FO – 70% VO (V) diets. **B.** Average methylation of 10 CpG positions within a CG island in the promoter of *fads2*. Different letters denote significant differences between treatments, no letters mean P>0.05.

In agreement, liver fatty acid profiles of juveniles obtained from broodstock with low *fads2* expression showed that products from Fads2 activity such as 20:2n-9 and 20:3n-6 were significantly reduced or did not changed such as 18:2n-9, 18:3n-6, 18:4n-3 and 20:4n-3, respect to juveniles from broodstock with high *fads2* expression and fed the V diet. Moreover, the substrates for Fads2 such as 20:1n-9, 20:2n-6, 18:1n-9, 18:3n-3 and 20:3n-3 did not change, or even increased, as it was the case for 18:2n-6. Both, these fatty acid profiles and our data on *fads2* expression suggest again a lower Fads2 activity in juveniles coming from the low *fads2* expression parents, in comparison to juveniles from broodstock with high *fads2* expression and fed the V diet. Nevertheless, as discussed above, the large variation in the *fads2* expression values did not allow detecting significant differences among juveniles from the different origins.

In fish, epigenetics has been a growing field of interest in the recent years. However, studies on epigenetic mechanisms are currently limited, partly due to limited availability of molecular tools for fish species. To our knowledge, this is the first study describing the promoter methylation pattern of some lipid metabolism related-genes (i.e. *fads2)* in gilthead sea bream. A previous study by (Xu *et al.*, 2014) in Japanese seabass showed that fish fed a n-3 LC-PUFA rich diet showed higher methylation in the promoter of *fads2,* while those fed VO showed around 4% lower methylation level. This increased methylation of the promoter region of *fads2* led to lower gene expression of *fads2* in negative correlation to methylation levels. These findings agree with the higher methylation at certain positions of the *fads2* promoter in the liver, and the lower liver content of fatty acids products of Fads2 activity found in the progeny of broodstock selected for low *fads2* expression. However, in our study, increased promoter methylation only reached 1.5%, and this could contribute to the high variation in the *fads2* expression of the progeny obtained from high *fads2* expression broodstock and consequently, to the lack of significance in the reduction of *fads2* expression. Studies in rats showed differential expression of the *Fads2* gene in negative correlation with the its promoter methylation. However, the change in methylation was higher in that study (up to 15% change in the methylation on -623 region of the promoter) (Hoile *et al.*, 2013) in comparison to what observed in the present study. Therefore, broodstock selection for low *fads2* expression, together with VO broodstock diets, caused epigenetic changes at

the *fads2* gene promoter, in the form of slight differences in cytosine methylation at certain positions that were reflected in the liver fatty acid profiles; products of Fads2 such as ARA, EPA and DHA were higher in the progenies obtained from low *fads2* selected fish than from high *fads2* parents. However, the gene expression of *fads2* between groups was similar in the present study, and high concentrations of ARA, EPA and DHA could be related to their selective retention in the liver, rather than to n-6 or n-3 LC-PUFA synthesis. Other data from the same individuals showed that muscle fatty acid content was not affected for ARA, EPA and DHA (Turkmen et al., submitted). Thus, further studies are needed to fully understand the relationship between *fads2* gene promoter methylation and corresponding gene expression.

Feeding broodstock with the ALA-rich diet (V diet) produced juveniles with reduced *elovl6* expression in the liver, particularly in those from broodstock with a low *fads2* expression. These results were in agreement with the 15-21% reduction in hepatic lipid contents and tendency to a lower content in saturated fatty acids. Besides, juveniles from broodstock fed the V diet also showed a reduction in *cpt1* expression. These results are in agreement with the down-regulation of *cpt1* in the liver of progenies from broodstock fed increased contents of dietary VO (Turkmen *et al.*, 2017) as discussed above. Indeed, in this previous study, a negative correlation was found between the level of VO inclusion in the broodstock diets and *cpt1* expression in the offspring juveniles when fed low FM and low FO diets (Turkmen *et al.*, 2017).

Likewise, feeding broodstock with the ALA rich diet (16.3% of the total fatty acid profile, V diet) produced juveniles with a reduced growth when challenged with the low FM and FO diet. Overall, these results suggest a nutritional programming effect of FO replacement by ALA, when the content of products of Fads2 (ARA, EPA and DHA) remain similar in the obtained eggs [LC-PUFA (% of total fatty acids), F diet: 20.29±2.70 and 18.44±4.02] (Turkmen *et. al.*, submitted). In previous studies, feeding gilthead sea bream broodstock a diet with 60% FO replacement by LO increased the ALA content in the eggs (13.08% of the total fatty acids) but reduced products of delta6 (ARA, EPA and DHA) was generated a nutritional programming effect by producing juveniles that grew better when fed low FM and low FO diets. However, higher (80% and 100%) FO replacement in broodstock diets negatively affected juveniles growth (Izquierdo *et al.*,

2015; Turkmen *et al.*, 2017). In these studies, 60% replacement of FO by VO also resulted in an increase in the amount of LC-PUFA precursors such as LA and ALA up to 73% and 1822% in eggs, but it was accompanied by a decrease in products of n-3 LC-PUFA biosynthesis such as EPA and DHA (33% and 36%, respectively) (Izquierdo *et al.*, 2015). However, in the present study, due to the contribution of the n-3 LC-PUFA fatty acids from the FM source used, the dietary levels of EPA and DHA changed to a lesser extent (15.5% and 23%, respectively) between both nutritional programming diets used, while the differences between LA and ALA were 76% and 1711%, respectively. Thus, in the present study, increase in precursors, but not a reduction in products of n-3 LC-PUFA synthesis, resulted in a programming effect evidenced as a reduction in the juvenile growth if challenged with low FM FO diets. There are a few studies regarding the effects of the increase in LA and/or total PUFA intake and its effects on the inhibition of the PUFA metabolic pathway. In previous studies, it was shown that high intake of 18C fatty acids and very low n-3 LC-PUFA levels might inhibit the *fads2* expression in gilthead sea bream (Izquierdo *et al.*, 2008; Izquierdo *et al.*, 2015). Moreover, studies in different species showed that the n-3 LC-PUFA DHA, but not EPA, was responsible for the down-regulation of *fads2* and *elovl2*-like elongase in livers of Atlantic salmon (Thomassen *et al.*, 2012). In addition, it has been observed that the ratio ALA to LA is important since it determines the percentage of DHA accumulation in plasma phospholipids (Gibson *et al.*, 2013). If the ratio of LA to ALA is low (0.5-0.8), as in the V diet of the present study, increasing levels of ALA up to 2% of dietary energy increases plasma DHA phospholipid, but conversely, this DHA levels decline sharply if ALA is above 6% of total energy (Gibson *et al.*, 2013).

The aim of this investigation was to assess the combined effects of broodstock selection and nutrition during the spawning period on some molecular mechanism involved in liver -key tissue of lipid biosynthesis-, as well as to examine the DNA methylation of the *fads2* promoter region in gilthead sea bream offspring. Broodstock of gilthead sea bream shows a huge variation of *fads2* expression at peripheral blood cells if fed with a high VM/VO diet before the spawning season. Selection of high *fads2* expression parents resulted in higher growth of the offspring. However, feeding the broodstock with ALA-rich diets negatively affected the offspring growth performance.

Some key genes for lipid biosynthesis in the liver, such as *elovl6* and *ppara*, were down-regulated in the offspring with the selection of the broodstock for low *fads2* expression, while the broodstock diet had a limited effect on the expression of analysed genes, although increased promoter methylation of the *fads2* gene in offspring originated from low *fads2* groups. Indeed, the small but statistically significant differences in the methylation of certain CpG positions within the *fads2* promoter did not affect *fads2* expression levels, and it might only mean that positions CpG2 and CpG3 are more permissive to change in methylation level because of their sequence context or other factors. Given than the diet of parents as a main effect did not affect the methylation at these positions, and that the strongest effects were observed for the *fads2* expression of parents (i.e. selection) and for selection by nutrition interaction, it can be speculated that the selection of parents in this study resulted in the segregation of epialleles (i.e., a specific DNA methylation pattern of a locus) with different susceptibility to change methylation at these positions. Thus, whether this and other CpG positions are involved in the transcriptional regulation of *fads2* under certain nutritional conditions or in particular genotypes remain to be elucidated. Studies focused more in detail on the effects of genetic selection of the broodstock by the *fads2* expression levels, the improvement of the nutritional programming diets, and the epigenetic effects on the progeny are in progress.

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Chapter 8

General Conclusions

1. As shown in Chapter 3 and Chapter 4, a FO replacement by LO equal or higher than 80% in gilthead seabream broodstock diets may have negative consequences for spawn quality, larval growth and even juveniles. Thus, FO replacement by LO up to 80–100% in broodstock diets for gilthead sea bream not only markedly reduce fecundity and spawn quality, but also the size of 45 dah and 4-month-old juveniles, as well as the *fads2* expression in larvae.
2. On the contrary, 60% replacement of FO by LO, leading to a reduction in LC-PUFA and an increase in the LA and ALA precursors, did not negatively affected offspring production, demonstrating the interesting potential of early nutritional programming of marine fish by broodstock feeding to improve long-term performance of the progeny (Chapter 3 and Chapter 4).
3. Parental feeding with FO replacement by VO induced long-term changes in PUFA metabolism, leading to an increased production of polyunsaturated fatty acids and the up-regulation of *fads2* expression, affecting also the expression of other lipid metabolism related genes (Chapter 3, Chapter 4, Chapter 5, Chapter 6, Chapter 7). For instance, replacement of FO with LO in parental diets affected offspring transcription of *lpl*, *cpt1* and *elov6,* genes related to regulation of energy metabolism in liver, allowing a better utilization of diets high in VO and VM.
4. In Chapter 3, this thesis provided the first evidences of the up-regulation of immune system-related genes in the offspring of seabream broodstock fed increased FO replacement by LO.
5. As demonstrated in Chapter 3 and Chapter 4 for the first time in fish nutrition, it is possible to nutritionally program marine fish, specifically gilthead seabream, offspring through modification of the fatty acid profiles of parental diets to improve the growth performance of juveniles fed low FO diets and even low FO and FM diets.

6. Despite, the moderate reduction of LC-PUFA and increase in LA and ALA precursors in broodstock diets improved offspring performance when challenged with a low FO and FM diet (Chapter 4), the sole increase of LA and ALA without LC-PUFA reduction did not improve offspring performance (Chapter 4). Thus, reduction of the n-3 LC-PUFA end products accompanied with 18C precursors seems to be required to nutritionally program the fish for better utilization of plant-based diets.
7. In Chapter 5, the persistent long-term effect of nutritional programming of gilthead sea bream through broodstock feeding and the efficiency of feeding a 'reminder' diet during juvenile stages to improve utilization of low-FM/FO diets.
8. Selection of gilthead sea bream broodstock with high *fads2* expression when fed a low FM and FO diet produces offspring that performs better, even when they are challenged with a low FM and FO diet. Besides, selection of high *fads2* broodstock induced regulation of genes such as *elovl6* and *cpt1* in juvenile stage if challenged with a very low FM FO diet (Chapter 6).
9. Finally, the results of this thesis showed that methylation of CpG islands in the *fads2* promotor, particularly in positions CpG2 and CpG3, are among the potential epigenetic mechanisms for regulation of gene expression in nutritionally programmed fish.

In summary, this thesis showed that nutritional programming of gilthead sea bream to better utilization of low FM and FO diets is possible using a balanced 18C and LC-PUFA diets provided during the spawning period that modifies the egg yolk content of the offspring. This improved utilization was persistent even after 16-months age if a low FM FO diet (reminder) was provided during the early juvenile stage at 3 months old. These improvements were observed as a higher growth of fish when challenged with low FM FO diets, as well as alterations in the gene expression patters of some lipid metabolism-related genes in the liver. Studies regarding the stress response of the offspring showed that high inclusion of VO diets in the broodstock diets showed negative effects on growth of the offspring and pro-inflammatory processes related genes were altered in early stages. Finally, besides nutritional programming, selection of broodstock selected with high *fads2* expression in peripheral blood cells allows to further improve

the spawning, larval and juvenile performance as well as the ability of the offspring to utilize low FM and low FO diets.

Chapter 9

Resumen ampliado en español

9.1 Importancia de n-3 LC-PUFA en nutricion humana

La investigación sobre la importancia del n-3 LC-PUFA (ácidos grasos poliinsaturados de cadena larga con 20 o más átomos de carbono) en la nutrición humana tiene una larga historia, habiéndose estudiado ampliamente el efecto de los n-3 LC-PUFA en la dieta humana. Aunque existen opiniones contradictorias, cada año más de 1000 publicaciones evidencian la importancia para la salud humana de los n-3 LC-PUFA, como el ácido eicosapentaenoico (EPA,20:5n-3) y el ácido docosahexaenoico (DHA,22:6n-3). Se ha descubierto que los n-3 LC-PUFA desempeñan un papel importante en la prevención de enfermedades cardiovasculares (ECV), hipertensión, diabetes, artritis, otros trastornos inflamatorios y autoinmunes, e incluso algunos tipos de cánceres (Simopoulos, 2000; Calder, 2018). Según la Organización Mundial de la Salud, las enfermedades cardiovasculares, como la cardiopatía isquémica y el infarto cerebrovascular, se encuentran entre las causas de muerte más importantes del mundo y representan una combinación de 15,2 millones de muertes (aproximadamente el 28% de todas las muertes) en 2016. (Health and Services, 2015). Existe una gran cantidad de literatura que muestra los efectos beneficiosos del consumo de LC-PUFA para la prevención de ECV, a pesar de que algunos estudios no han podido establecer una relación clara entre el tratamiento con n-3 LC-PUFA y la mortalidad por ECV (Abdelhamid *et al.*, 2018). Algunos alimentos terrestres como las nueces, el aceite de linaza, el aceite de soja o la colza son ricos en ácidos grasos 18C, precursores de n-3 LC-PUFA, como el ácido α-linolénico (ALA, 18: 3n-3). Sin embargo, ninguno de ellos tiene cantidades considerables de n-3 LC-PUFA (Whelan and Rust, 2006). Los seres humanos tienen cierta capacidad para sintetizar el n-3 LC-PUFA de cadena larga a partir del ALA (Domenichiello *et al.*, 2015), aunque esta capacidad es restringida. Así, se calcula que la relación de converción de ALA a EPA es de 0.2% en el caso del EPA e incluso menor, alrededor del 0.1% para el DHA (22: 6n-3). Sin embargo, esta proporción alcanzar niveles del 6% en EPA en el caso de mujeres embarazadas, pudiendo satisfacer

las demandas requerida por el embarazo (Burdge, 2006). Además del género, los ratios de conversión pueden verse influenciados por una variedad de otros factores como la edad, las hormonas y los antecedentes genéticos (Bolton-Smith *et al.*, 1997; Vessby *et al.*, 2002; Koletzko *et al.*, 2010).

En la mayoría de las regiones geográficas, los productos derivados del mar, particularmente los pescados azules, son la única fuente de ácidos grasos poliinsaturados de cadena larga (AGPI-CL) y, por lo tanto, el consumo de pescado está siendo respaldado por las diferentes autoridades sanitarias (SACN, 2004; Health and Services, 2015). En este sentido, las autoridades sanitarias del Reino Unido recomiendan un consumo diario de 450 mg n-3 LC-PUFA. Mientras que una porción de 100 g de pollo suministra solo 0.06 g de EPA, DPA, DHA, la misma cantidad de salmón proporciona 2.2 gramos de estos ácidos grasos (Calder, 2018) siendo por tanto recomendado el consumo de pescado. Teniendo en cuenta que en la actualidad uno de cada dos productos acuáticos consumidos en el mundo proviene de la acuicultura, este sector de producción de alimentos tiene una responsabilidad principal en el suministro de n-3 LC-PUFA para la demanda de los consumidores. A pesar de que el pescado procedente de la acuicultura tiene un mayor contenido de n-3 LC-PUFA que el pescado salvaje, debido a que los perfiles de ácidos grasos de filete dependen de la dieta de los peces, resulta del todo indispensable suministrar a estas dietas estos valiosos ácidos grasos. Sin embargo, el aceite de pescado (FO) es la fuente principal en el alimento para peces, pero no fácilmente sostenible ya que su reemplazo por aceites vegetales (VO) puede afectar negativamente el valor nutricional del filete de pescado para consumo humano (Izquierdo *et al.*, 2003, Castro et al., 2015; Izquierdo *et al.*, 2005; Torstensen *et al.*, 2010).

9.2 Importancia del n-3 LC-PUFA en peces

En términos generales, los peces marinos parecen tener escasa capacidad para utilizar aceites terrestres debido a sus altos requerimientos de n-3 LC-PUFA y a la actividad insuficiente de las enzimas que sintetizan n-3 LC-PUFA a partir de los precursores de ácidos grasos que se encuentran en VO. Por lo tanto, la sustitución de FO por VO parece más desafiante en los peces marinos que en otras especies que están mejor adaptadas para utilizar de manera más eficiente los lípidos de la dieta. La mayor

capacidad de biosíntesis de LC-PUFA en peces de agua dulce en comparación con los peces marinos podría estar relacionada con las diferencias en los hábitos alimenticios y la ingesta de nutrientes, ya que los peces marinos tienen un acceso continuo a fuentes ricas en LC-PUFA a lo largo de su vida (Sargent *et al.*, 1995 ; Tocher, 2003). Además, estas diferencias entre especies de peces se han relacionado con la evolución diversa de ciertos genes involucrados en la biosíntesis de lípidos (Castro *et al.*, 2016). El ácido linoleico (LA, 18:2n-6) y ALA (18:3n-3) son los precursores de la biosíntesis de n-3 LC-PUFA y se pueden convertir en ácidos grasos de cadena de carbono más largos, como el EPA (20:5n-3) y DHA (22:6n-3) después de varias reacciones de elongación y desaturación (Figure 9-1). El primer paso de la síntesis de n-3 LC-PUFA en vertebrados se logra mediante la enzima faty acyl delta-6-desaturasa (delta-6), codificada por el gen de la 2 desaturasa del ácido graso (*fads2*), al introducir un doble enlace en una posición específica de ácidos grasos de cadena larga. En el primer paso de esta ruta, LA (18: 2n-6) y ALA (18:3n-3) se pueden convertir a 18: 3n-6 y 18: 4n-3, respectivamente. Después de una etapa de alargamiento, estos ácidos grasos pueden convertirse a 20:3n-6 y 20:4n-3. De estos precursores, hay otra etapa de desaturación catalizada por una enzima acil delta-5-desaturasa (delta-5) involucrada en la conversión a 20:5n-3 (EPA) y 20:4n-6 (ARA) (Figura 1 1). Hasta la fecha, solo hay un gen desaturasa (*fads2*) aislado de dorada y se ha encontrado una actividad delta-5 muy baja durante los estudios in vitro (Tocher and Ghioni, 1999). Las desaturasas de peces han evolucionado de manera diferente dependiendo de la especie, mostrando gran variedad (Castro *et al.*, 2016). Por ejemplo, en el pez cebra (Danio rerio) el gen *fads2* da lugar a las actividades delta-5 y delta-6 (Hastings *et al.*, 2001). Bajo esta perspectiva, algunas vías metabólicas y la capacidad de síntesis de LC-PUFA n-3 en dorada no están claras. Sin embargo, con el conocimiento y la disponibilidad de las actuales herramientas genómicas, incluida la reciente publicación de la secuencia completa del genoma de dorada (Pauletto, 2018 #1754), nuevas oportunidades para comprender mejor los mecanismos de síntesis de LC-PUFA n-3 y su regulación se están abriendo para responder a las limitaciones del metabolismo de LC-PUFA n-3 en dorada.

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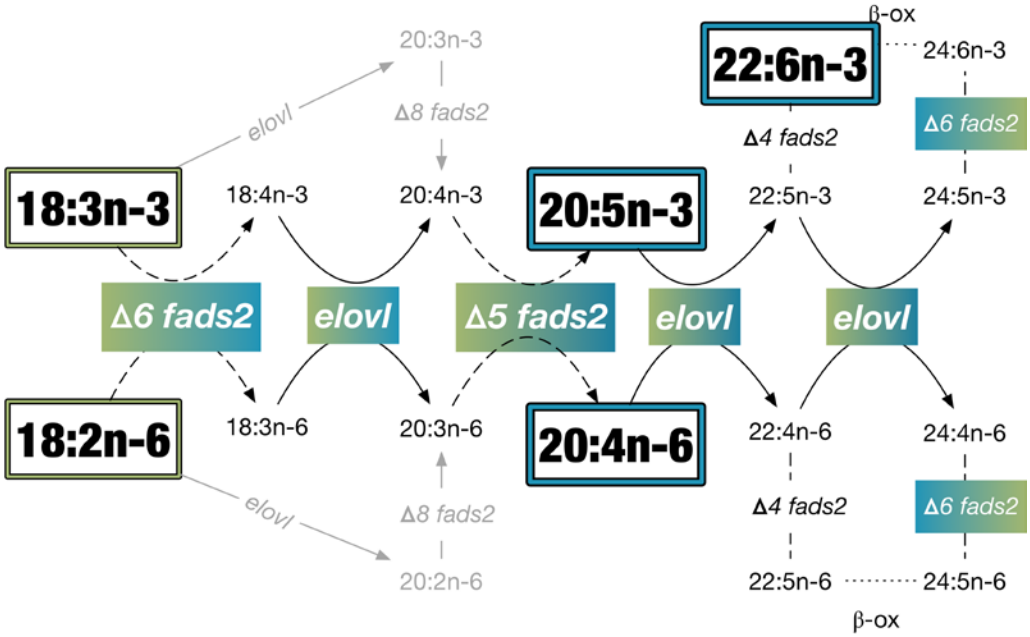

Figure 9-1 | Vía de biosíntesis de LC-PUFA y las enzimas involucradas

9.2.1 Los genes relacionados con el metabolismo LC-PUFA estudiados en la presente tesis

Como se indicó anteriormente la actividad delta-6 es la de una enzima limitante de la velocidad de la biosíntesis de LC-PUFA y está codificada por *fads2* y, siendo por esta razón, un gen ampliamente estudiado en diversos organismos y especies de peces. El papel crucial de *fads2* está claro en la biosíntesis de LC-PUFA n-3, sin embargo, hay varias otras enzimas involucradas en esta vía de biosíntesis. Así, la síntesis de LC-PUFA también requiere elongación de la cadena catalizada por elongasas (elovl) con diferentes preferencias de sustrato (Monroig *et al.*, 2011). Entre ellos, el alargamiento de los ácidos grasos de cadena muy larga, la proteína 6 (elovl6) es una enzima lipogénica clave que alarga los ácidos grasos saturados de cadena larga y monoinsaturados de 12, 14 y 16 átomos de carbono, que ha recibido mucha atención debido a su importancia en los trastornos metabólicos en mamíferos (Matsuzaka and Shimano, 2009). Además, el LC-PUFA puede tener un efecto directo en la expresión de otros genes relacionados con el metabolismo de los lípidos o los carbohidratos (Clarke, 2001). La lipoproteína lipasa (lpl) facilita la absorción tisular de los ácidos grasos circulantes (Bell and Koppe, 2010) de las lipoproteínas y su expresión en el hígado puede ser regulada por n-3 LC-PUFA (Raclot *et al.*, 1997). La provisión de energía se realiza mediante la β-oxidación de los ácidos grasos libres transportados a las mitocondrias en forma de ésteres acil-carnitina

por carnitina aciltransferasas, como la carnitina palmitoiltransferasas (Sargent *et al.*, 2002). El reemplazo de FO con VO cambia la composición de ácidos grasos del hígado y el músculo, afectando la capacidad de β oxidación y regulando la expresión de los genes tanto de la a carnitina palmitoiltransferasa I (cptI) como de la carnitina palmitoiltransferasa II (*cptII*) (Kjaer *et al.*, 2008; Leaver *et al.*, 2008; Vestergren *et al.*, 2012; Xue *et al.*, 2015). La β oxidación también tiene lugar en el peroxisoma y está modulada por los receptores activadores del proliferador de peroxisoma (PPAR). En dorada se han caracterizado tres isoformas de PPAR diferentes (α, β, γ), siendo la forma principal expresada en el hígado el receptor alfa (ppara) activado por el proliferador de peroxisoma (Leaver *et al.*, 2008). Los PPAR son receptores nucleares que regulan la diferenciación, el crecimiento y el metabolismo, habiéndose descrito en mamíferos mecanismos epigenéticos para regular estos procesos en los que se encuentran involucradas todas las isoformas de los PPAR (Corbin, 2011). Por ejemplo, alimentar a ratas preñadas con una dieta restringida en proteínas reduce la metilación del promotor ppara en la descendencia y esta hipometilación persiste hasta la edad adulta (Lillycrop *et al.*, 2008). Finalmente, otro gen potencialmente regulado por LC-PUFA es la ciclooxigenasa-2 (cox2), una enzima clave en la biosíntesis de prostanoides (Ishikawa and Herschman, 2007).

9.3 Concepto de programación nutricional en mamíferos y otros vertebrados

La programación nutricional se describe como los "eventos durante períodos críticos o sensibles de desarrollo que pueden" programar "la estructura o función a largo plazo o durante toda la vida del organismo" (Lucas, 1998). El concepto de "programación nutricional" supone que las alteraciones del medio ambiente durante las etapas tempranas de la vida de un organismo afectan su fenotipo en etapas posteriores de la vida. Los cambios ambientales durante los primeros períodos críticos, como la embriogénesis o el desarrollo temprano (denominados períodos de plasticidad del desarrollo), proporcionan una herramienta al organismo para pronosticar posibles desafíos ambientales en el futuro y brindan la oportunidad de ajustar su metabolismo para una mejor adaptación y aumentar sus posibilidades. Para sobrevivir y reproducirse. En cierto modo, estos períodos críticos de plasticidad del desarrollo permiten que el organismo se prepare para su entorno futuro. Estos resultados a largo plazo pueden

abordarse de tres formas diferentes: daño directo; inducción, supresión o desarrollo deficiente de una estructura somática resultante del estímulo o injuria durante un período crítico; "ajuste" fisiológico por un estímulo o injuria temprana en un período crítico, con consecuencias a largo plazo para la función. Por ejemplo, la descendencia que enfrentó restricciones calóricas durante el crecimiento fetal puede adoptar un fenotipo descrito como "fenotipo ahorrativo" más adelante en la vida, con cambios permanentes en el metabolismo de la glucosa-insulina (Hales and Barker, 2001).

Hay varias evidencias que muestran cómo el organismo se prepara a sí mismo si enfrenta restricciones de energía durante el desarrollo fetal. Una de las primeras evidencias del vínculo entre la nutrición temprana y sus efectos a largo plazo en humanos es el conocido efecto de la hambruna holandesa de la Segunda Guerra Mundial. Durante este bloqueo de alimentos en los Países Bajos, la ingesta de calorías de los individuos disminuyó de 1800 kcal / día en diciembre de 1943, por debajo de 1000 kcal a fines de noviembre y de solo 400 a 800 kcal / día en abril de 1945. Así, durante su período, junto con el resto de la población, las mujeres embarazadas se enfrentaron a una grave hambruna que impuso restricciones de calorías obligatorias a sus embriones durante las primeras etapas de su vida. Los registros bien guardados durante la Segunda Guerra Mundial permitieron a los investigadores analizar los efectos de esta hambruna y la relación entre la mala nutrición materna y la salud de los hijos. Además, investigadores actuales han podido estudiar diferentes parámetros en su descendencia, proporcionando resultados de un experimento humano casi perfectamente diseñado en relación a los efectos de la restricción nutricional materna y los resultados posteriores en la progenie. Este estudio fue uno de los primeros ejemplos del creciente campo de los orígenes del desarrollo de la salud y la enfermedad en los seres humanos, que muestran que una nutrición inadecuada durante los procesos de desarrollo puede tener diferentes consecuencias metabólicas en la descendencia a través de mecanismos epigenéticos (Barella *et al.*, 2017).

Estos cambios de adaptación se pueden utilizar para obtener individuos que se comporten de mejor manera a la hora de utilizar los alimentos en los sistemas de producción animal. Usualmente, en los sistemas de producción, las dietas están formuladas de manera balanceada suministrando los componentes nutricionales

necesarios, seleccionando los ingredientes adecuados en función de sus características nutricionales, costo y disponibilidad. Por lo tanto, las restricciones en la disponibilidad de un alimento de alta calidad conducen a la búsqueda de ingredientes alternativos.

En cuanto a los peces se refiere, éstos a lo largo de la evolución se han adaptado a la presa en ciertos tipos de organismos, ajustando su metabolismo al uso de los nutrientes que contienen dichas presas. Basándose en estas premisas, las dietas en acuicultura se formularon lo más cerca posible de esas presas para suministrar los nutrientes necesarios para lograr un crecimiento óptimo y un buen estado de salud.

9.3.1 Importancia de la acuicultura de dorada

La dorada (*Sparus aurata*) es una especie importante para la acuicultura europea. Aunque la producción de dorada es más intensa en el mar Mediterráneo, también existe en los océanos Atlántico e Índico, así como en las aguas interiores de África (FAO, 2018). La producción acuícola de la dorada representa casi 190000 toneladas en 2016 (FAO, 2018) (Figure 9-2), manteniéndose la producción acuícola en un crecimiento constante desde 2012 hasta 2016 (FAO, 2018).

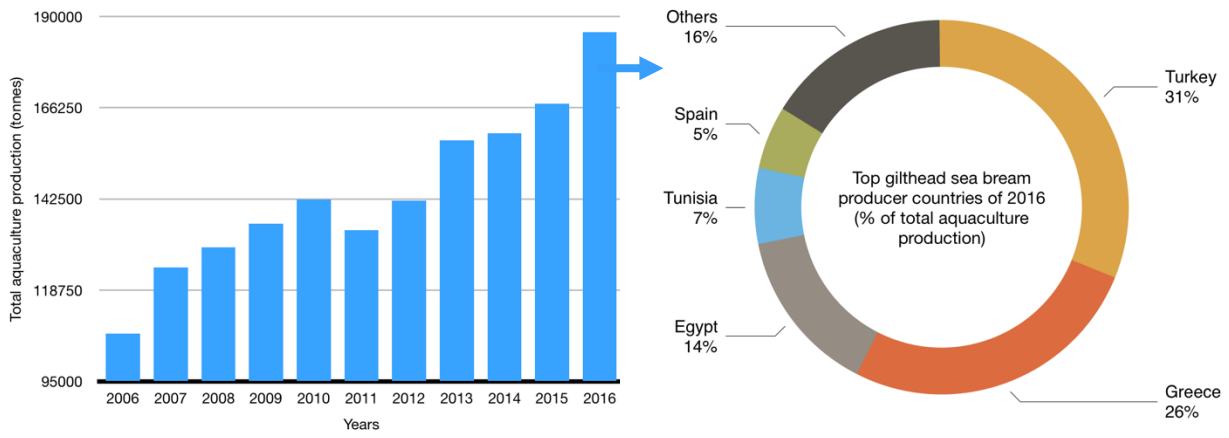

Figure 9-2 | La producción de dorada en los últimos diez años con los datos disponibles de FAO 2018, el gráfico circular muestra el % de contribución de la producción total de 2016

Esta especie, junto con la lubina europea, es una de las principales especies marinas en la acuicultura mediterránea. Según los datos más recientes (2016), Turquía es el principal productor con el 31% de la producción acuícola total, seguido por Grecia y Egipto con el 26% y el 14% de la producción total respectivamente (FAO, 2018) (Figure 9-2).

9.4 Objetivos

El costo de la alimentación es uno de los principales gastos de mantenimiento en la acuicultura, el suministro de alimentos económicamente sostenibles es de suma importancia. Tradicionalmente, la FM y el FO eran fuentes básicas de proteínas y lípidos en las dietas de peces de aleta, pero las restricciones en su disponibilidad y el aumento en los precios han limitado seriamente su uso en alimentos acuáticos. Esto ha obligado a la industria de producción de piensos a buscar alternativas más sostenibles y económicas. Si bien los aceites de origen marino son ricos en LC-PUFA n-3 (esenciales para que los peces marinos promuevan el crecimiento y mantengan un buen estado de la salud), los VO carecen de estos ácidos grasos pero son ricos en ácidos grasos n-6 y n-9 del 18C. En términos generales, los peces marinos tienen un requisito estricto de PUFA, incluidos ARA (20:4n-6), EPA (20:5n-3) y DHA (22:6n-3) (Sargent *et al.*, 1999). En consecuencia, los peces marinos parecen tener una menor capacidad para utilizar aceites terrestres debido a sus altos requerimientos de LC-PUFA n-3 y la actividad insuficiente de las enzimas que sintetizan LC-PUFA n-3 a partir de los precursores de ácidos grasos que se encuentran en el VO. Por lo tanto, la sustitución de FO con VO parece más desafiante que en otras especies que están mejor equipadas para utilizar los lípidos de la dieta de manera más eficiente. El concepto de programación temprana plantea la posibilidad interesante de dirigir vías o funciones metabólicas específicas en peces juveniles. Por ejemplo, la programación nutricional puede mejorar el uso de sustitutos de FM y FO y, por lo tanto, promover la sostenibilidad en la acuicultura al maximizar la capacidad de utilización de lípidos en los peces. Así, los principales objetivos de esta tesis son:

1. Investigar el valor potencial de los ácidos grasos como moduladores de la programación nutricional temprana y determinar el efecto de la alimentación de los padres con dietas VO en el rendimiento de la descendencia a lo largo de su vida, centrándose en la calidad del huevo, el crecimiento de larvas y juveniles. Se han realizado dos ensayos diferentes a largo plazo y son presentados en el Capítulo 3 y el Capítulo.
2. Establecer el efecto de la alimentación parental con dietas VO en el metabolismo de los lípidos de la descendencia, analizando la composición de los ácidos grasos

y proximales y la expresión de genes seleccionados en huevos, larvas y juveniles (Capítulo 3 y Capítulo 4).

3. Analizar el efecto de la alimentación parental con dietas VO en la expresión de genes seleccionados relacionados con la salud y la resistencia al estrés de los descendientes (Capítulo 3).
4. Estudiar el efecto de la alimentación de los padres con dietas VO en la capacidad de los descendientes juveniles para usar dietas con alto VO. Para ese propósito, los juveniles fueron desafiados con dietas bajas en FO (Capítulo 3).
5. Determinar el efecto de la alimentación parental con dietas VO en la capacidad de los descendientes para usar dietas altas en VO y VM. Este objetivo se abordó en dos ensayos de alimentación con jóvenes descendientes alimentados con una dieta baja en FF y baja en FM (Capítulo 4 y Capítulo 6).
6. Averiguar si existe una persistencia de los efectos de la programación nutricional a través de la alimentación de los padres en la descendencia al comienzo del desarrollo de la primera gónada y si esto afecta la calidad del filete para el consumidor. Para abordar este objetivo, se han investigado los efectos de la alimentación de los reproductores y un desafío nutricional temprano en la etapa juvenil sobre el crecimiento, la composición química y de los ácidos grasos del músculo y el hígado, y la expresión de genes seleccionados en el hígado (Capítulo 5).
7. Investigar si existe un efecto combinado del acondicionamiento nutricional de los reproductores y el desafío nutricional de los juveniles en la capacidad de los descendientes para usar dietas altas en VO y VM (Capítulo 5).
8. Determinar la interacción potencial entre la selección de peces con una mayor expresión de *fads2* y la programación nutricional a través de la nutrición de los reproductores. Para ese propósito, los reproductores de dorada se seleccionaron para respuesta alta o baja *fads2* y se alimentaron con dietas altas en FO o en VO , investigándose el rendimiento de sus hijos y la utilización de una dieta baja en FM y FO (Capítulo 6).
9. Comprender mejor los posibles mecanismos epigenéticos implicados en la programación nutricional de la dorada a través del acondicionamiento de los

reproductores. Para ese propósito, en el Capítulo 7 se estudió la potencial metilación del ADN de promotores de genes específicos.

9.5 Resumen de los experimentos

9.5.1 Capítulo 3. Los perfiles de LC-PUFA en las dietas parentales indujeron efectos a largo plazo sobre el crecimiento, los perfiles de ácidos grasos y la expresión de genes relacionados con el sistema inmunitario de PUFA y los genes relacionados con el sistema inmunitario en la cría de dorada

La programación nutricional del pescado puede contribuir a optimizar sus contenidos en n-3 LC-PUFA, que son ácidos grasos importantes para la salud humana. El presente estudio investigó los efectos de la programación nutricional a través de la alimentación de los padres en el rendimiento de los descendientes y la expresión de genes seleccionados relacionados con la resistencia al estrés. Las doradas se alimentaron con dietas que contenían varias proporciones de aceite de pescado (FO) / aceite vegetal (VO) para determinar sus efectos sobre el rendimiento de las crías a lo largo de la embriogénesis, el desarrollo larvario y los periodos de crecimiento juvenil. El aumento de la sustitución de FO en la dieta por aceite de linaza (LO) hasta un 80% LO redujo significativamente el número total de huevos producidos por kg / hembra / por desove. Además, a las 30 h, la alimentación de los padres con un aumento de LO llevó a un aumento de la regulación de las *fads2* que se correlacionó con el aumento en las tasas de conversión de los PUFA relacionados. Además, la expresión de los genes cox2 y tnf-a también fue regulada al alza por el aumento de LO en las dietas parentales hasta 60% u 80%, respectivamente. Sin embargo, durante el desafío nutricional, el crecimiento más bajo se encontró en los juveniles de reproductores alimentados con 100% de FO. El aumento en los niveles de LO en la dieta de reproductores hasta 60LO aumentó el LC-PUFA en los juveniles, independientemente de la dieta de los mismos. Los resultados mostraron que es posible programar nutricionalmente las crías de dorada a través de la modificación de los perfiles de ácidos grasos de las dietas parentales para mejorar el rendimiento de crecimiento de los juveniles alimentados con dietas bajas en FO, induciendo cambios a largo plazo en el metabolismo de los AGPI en relación con la regulación al alza de expresión *fads2*. Este estudio proporcionó las primeras evidencias

de la regulación al alza de los genes relacionados con el sistema inmunológico en la descendencia de los padres alimentados con un mayor reemplazo de FO por LO.

9.5.2 Capítulo 4. Programación nutricional a través de dietas de reproductores para mejorar la utilización de dietas muy bajas en harina de pescado y aceite de pescado en dorada (Sparus aurata)

La eliminación completa de la harina de pescado (FM) y el aceite de pescado (FO) es necesaria para promover el desarrollo sostenible de la acuicultura y, para ello, es necesario el pescado de alta calidad y rápido crecimiento que se alimenta sin FM y FO. La programación nutricional temprana puede permitir la producción de pescado mejor adaptado para utilizar dietas con alimentos vegetales (VM) y aceites (VO). El objetivo principal de este estudio fue investigar el valor potencial de los ácidos grasos como moduladores de la programación nutricional temprana en peces marinos para una mejor utilización de VO / VM. Para ese propósito, los reproductores de dorada (*Sparus aurata*) fueron alimentados con cuatro niveles diferentes de reemplazo de FO por el aceite de linaza (LO) y su efecto sobre la fecundidad y la calidad del desove, la composición del huevo, la expresión del gen de la Δ-6-desaturasa (6D), en la progenie El rendimiento de crecimiento y su respuesta de crecimiento a un desafío con dietas bajas en FO y FM, pero altas en VO y VM. Los resultados mostraron que la alimentación de reproductores de dorada con dietas altas en LO tuvo efectos a largo plazo en la progenie. Por lo tanto, el reemplazo de FO por LO hasta 80–100% en dietas de reproductores para dorada no solo redujo la fecundidad y la calidad de reproducción, sino también el crecimiento de juveniles de 45 dah y 4 meses, así como la expresión del gen Δ6D. Sin embargo, cuando los juveniles de 4 meses de edad fueron alimentados con una dieta baja en FM y FO, incluso los de reproductores alimentados con solo un 60% de reemplazo de FO por LO mostraron un mayor crecimiento y utilización de alimento que los juveniles de padres alimentados con FO. Estos resultados demuestran el interesante potencial de la programación nutricional temprana de peces marinos mediante la alimentación de reproductores para mejorar el rendimiento a largo plazo de la progenie. Se están realizando estudios adicionales para determinar los niveles óptimos de nutrientes en las dietas de los reproductores y los mecanismos moleculares implicados para desarrollar estrategias efectivas de intervención nutricional para esta especie.

9.5.3 Capítulo 5. La programación nutricional de los padres y un recordatorio durante la etapa juvenil afectan el crecimiento, el metabolismo de los lípidos y la utilización en etapas posteriores del desarrollo en el teleósteo marino, la dorada (*Sparus aurata*)

La nutrición durante la periconcepción y el desarrollo temprano pueden modular las rutas metabólicas para preparar a las crías para condiciones adversas a través de un proceso conocido como programación nutricional. En la dorada, el reemplazo del aceite de pescado (FO) con aceite de linaza (LO) en las dietas de reproductores mejora el crecimiento en las crías de 4 meses de edad con dietas bajas en FO y de harina de pescado (FM) durante 1 mes. El presente estudio se investigó más a fondo los efectos de la alimentación de los reproductores en la misma descendencia cuando tenían 16 meses de edad y fueron desafiados por segunda vez con la dieta baja en FM y baja en FO durante 2 meses. Los resultados mostraron que la sustitución de la alimentación parental moderada con FO con LO, combinada con la alimentación juvenil a los 4 meses de edad con dietas bajas en FM y bajas en FF, mejoró significativamente ($P < 0.05$) el crecimiento de la descendencia y la utilización de alimento en dietas baja FM / FO incluso cuando tenían 16 meses de edad: es decir, cuando estaban al borde de su primera temporada reproductiva. La composición de los ácidos grasos del hígado se vio significativamente afectada por las dietas AGPI de reproductores, así como por su interacción. Además, la reducción de los ácidos de cadena larga y el aumento del ácido α-linolénico y el ácido linoleico en las dietas de reproductores conducen a una importante disminución de la lipoproteína lipasa hepática ($P < 0.001$) y el alargamiento de la proteína de ácidos grasos de cadena muy larga ($P<0.01$). Además, los valores de ácido graso 2 desaturasa se correlacionaron positivamente con niveles hepáticos de 18:4n-3, 18:3n-6, 20:5n-3, 22:6n-3 y 22:5n-6. Por lo tanto, este estudio demostró la programación nutricional a largo plazo de dorada a través de la alimentación de los reproductores, el efecto de alimentar una dieta 'recordatoria' durante las etapas juveniles para mejorar la utilización de dietas bajas en FM / FO y el crecimiento de peces, así como la regulación de expresión génica a lo largo del ciclo de vida de los peces.

9.5.4 Capítulo 6. La selección de reproductores y la programación nutricional con ALA (18:3n-3) dieta rica afecta el rendimiento de crecimiento de la descendencia en dorada (*Sparus aurata*)

La eliminación completa de la harina de pescado (FM) y el aceite de pescado (FO) es necesaria para promover el desarrollo sostenible de la acuicultura y, para ello, es necesario el pescado de alta calidad y rápido crecimiento que se alimenta sin FM y FO. La programación nutricional temprana puede permitir la producción de pescado mejor adaptado para utilizar dietas con alimentos vegetales (VM) y aceites (VO). El objetivo principal de este estudio fue investigar el valor potencial de los ácidos grasos como moduladores de la programación nutricional temprana en peces marinos para una mejor utilización de VO / VM. Para ese propósito, los reproductores de dorada (*Sparus aurata*) fueron alimentados con cuatro niveles diferentes de reemplazo de FO por el aceite de linaza (LO) y su efecto sobre la fecundidad y la calidad del desove, la composición del huevo, la expresión del gen de la fatty acyl desaturasa (*fads2*), en la progenie El rendimiento de crecimiento y su respuesta de crecimiento a un desafío con dietas bajas en FO y FM, pero altas en VO y VM. Los resultados mostraron que la alimentación de reproductores de dorada con dietas altas en LO tuvo efectos a largo plazo en la progenie. Por lo tanto, el reemplazo de FO por LO hasta 80–100% en dietas de reproductores para dorada no solo redujo la fecundidad y la calidad de reproducción, sino también el crecimiento de juveniles de 45 dah y 4 meses, así como la expresión del gen *fads2*. Sin embargo, cuando los juveniles de 4 meses de edad fueron alimentados con una dieta baja en FM y FO, incluso los de reproductores alimentados con solo un 60% de reemplazo de FO por LO mostraron un mayor crecimiento y utilización de alimento que los juveniles de padres alimentados con FO. Estos resultados demuestran el interesante potencial de la programación nutricional temprana de peces marinos mediante la alimentación de reproductores para mejorar el rendimiento a largo plazo de la progenie. Se están realizando estudios adicionales para determinar los niveles óptimos de nutrientes en las dietas de los reproductores y los mecanismos moleculares implicados para desarrollar estrategias efectivas de intervención nutricional para esta especie.

9.5.5 Capítulo 7. La selección y nutrición de los reproductores afectan los genes relacionados con el metabolismo de los lípidos y la metilación del promotor del gen de la desaturasa 2 del ácido graso (*fads2*) en la cría de la dorada.

Investigaciones anteriores han demostrado que los ácidos grasos poliinsaturados (PUFA) en las primeras etapas de desarrollo desempeñan un papel clave en la regulación del metabolismo de LC-PUFA n-3 de la descendencia en mamíferos y otros animales, como los peces. Sin embargo, no se comprende bien si la regulación se debe a una suplementación inadecuada de los precursores (18:2n-6, LA 18:3n-3, ALA) y / o los productos (20:4n-6, 20:5n- 3, 22:6n-3) de la síntesis de PUFA de cadena larga n-3 en las dietas parentales que modifica los nutrientes disponibles durante la embriogénesis. Además, un mecanismo epigenético como la metilación del promotor del gen *fads2*, que codifica una enzima limitante de la velocidad en LC-PUFA aún no se ha estudiado en detalle en dorada. Además, aún no se ha investigado el potencial de selección de los padres utilizando los niveles de expresión de *fads2* en las células de la sangre periférica como un biomarcador en los padres antes del período de desove. Para probar estos factores, el presente estudio consistió en tres fases, en la primera fase se seleccionaron los reproductores en función de su expresión *fads2* y en la segunda fase, cada reproductor se alimentó con una dieta rica en ALA para inducir la programación nutricional o una dieta de control. Por último, en la tercera fase, la progenie obtenida de estos grupos fue desafiada con una dieta de desafío en la etapa juvenil. Este estudio mostró que la selección por la expresión *fads2* puede mejorar la utilización de las dietas bajas en n-3 LC-PUFA de las crías, sin embargo, una dieta rica en ALA durante la embriogénesis causa efectos negativos en el crecimiento de las crías. Incluso los peces fueron alimentados con la misma dieta, el análisis epigenético mostró, la selección y el tipo de la dieta de reproductores causó modificaciones en la metilación del promotor de *fads2*.

9.6 Conclusiones

1. Como se muestra en el Capítulo 3 y el Capítulo 4, un reemplazo de FO por LO igual o superior al 80% en las dietas de reproductores de dorada puede tener consecuencias negativas para la calidad del desove, el crecimiento larvario e incluso los juveniles. Por lo tanto, el reemplazo de FO por LO hasta 80–100% en

dietas de reproductores para dorada no solo reduce notablemente la fecundidad y la calidad de reproducción, sino también el tamaño de los juveniles de 45 dah y 4 meses de edad, así como la expresión *fads2* en larvas.

2. Por el contrario, el reemplazo del 60% de FO por LO, lo que llevó a una reducción de LC-PUFA y un aumento en los precursores de LA y ALA, no afectó negativamente la producción de la descendencia, lo que demuestra el interesante potencial de la programación nutricional temprana de los peces marinos. por la alimentación de los reproductores para mejorar el rendimiento a largo plazo de la progenie (Capítulo 3 y Capítulo 4).
3. La alimentación de los padres con un reemplazo de FO por VO indujo cambios a largo plazo en el metabolismo de los AGPI, lo que condujo a un aumento de la producción de ácidos grasos poliinsaturados y al aumento de la expresión de *fads2*, afectando también la expresión de otros genes relacionados con el metabolismo de los lípidos (Capítulo 3, Capítulo 4, Capítulo 5, Capítulo 6, Capítulo 7). Por ejemplo, el reemplazo de FO con LO en las dietas parentales afectó a la transcripción de *lpl*, *cpt1* y *elovl6* de los descendientes, genes relacionados con la regulación del metabolismo energético en el hígado, lo que permite una mejor utilización de las dietas altas en VO y VM.
4. En el Capítulo 3, esta tesis proporcionó las primeras evidencias de la regulación al alza de los genes relacionados con el sistema inmunitario en la descendencia de reproductores de dorada alimentados con un mayor reemplazo de FO por LO.
5. Como se demostró en el Capítulo 3 y en el Capítulo 4 por primera vez en nutrición de peces, es posible programar nutricionalmente peces marinos, específicamente doradas, a través de la modificación de los perfiles de ácidos grasos de las dietas parentales para mejorar el rendimiento de crecimiento de los juveniles alimentados. Dietas bajas en FO e incluso dietas bajas en FO y FM.
6. A pesar de la reducción moderada de LC-PUFA y el aumento de los precursores de AL y ALA en las dietas de reproductores, se mejoró el rendimiento de los descendientes cuando se enfrentó a una dieta baja en FO y FM (Capítulo 4), el único aumento de LA y ALA sin reducción de LC-PUFA no mejoró el rendimiento de la descendencia (Capítulo 4). Por lo tanto, la reducción de los productos finales LC-PUFA n-3 acompañados con precursores de 18C parece ser necesaria para

programar nutricionalmente el pescado para una mejor utilización de las dietas basadas en vegetales.

7. En el capítulo 5, existe efecto persistente a largo plazo de la programación nutricional de la dorada a través de la alimentación de los reproductores y la eficiencia de la alimentación de una dieta "recordatoria" durante las etapas juveniles para mejorar la utilización de las dietas con bajo contenido de FM / FO
8. La selección de reproductores de dorada con alta expresión *fads2* cuando se alimenta con una dieta baja en FM y FO produce crías que se desempeñan mejor, incluso cuando se enfrentan a una dieta baja en FM y FO. Además, la selección de reproductores con alto contenido en *fads2* indujo la regulación de genes como *elovl6* y *cpt1* en la etapa juvenil si se desafiaba con una dieta muy baja en FM FO (Capítulo 6).
9. Finalmente, los resultados de esta tesis mostraron que la metilación de las islas CpG en el promotor *fads2*, particularmente en las posiciones CpG2 y CpG3, se encuentran entre los posibles mecanismos epigenéticos para la regulación de la expresión génica en peces nutricionalmente programados.

En resumen, esta tesis demostró que la programación nutricional de dorada para una mejor utilización de las dietas bajas en FM y FO es posible utilizando dietas balanceadas con 18C y LC-PUFA proporcionadas durante el período de desove que modifica el contenido de la gota lipídica de la descendencia. Esta mejor utilización fue persistente incluso después de los 16 meses de edad si se proporcionó una dieta baja en FM (recordatoria) durante la etapa temprana de la edad juvenil a los 3 meses de edad. Estas mejoras se observaron como un mayor crecimiento de los peces cuando se desafió con dietas bajas en FM, así como alteraciones en los patrones de expresión génica de algunos genes relacionados con el metabolismo de los lípidos en el hígado. Los estudios sobre la respuesta al estrés de la descendencia mostraron que la alta inclusión de las dietas VO en las dietas de reproductores mostró efectos negativos sobre el crecimiento de la descendencia y los genes relacionados con los procesos pro inflamatorios se alteraron en las etapas iniciales. Finalmente, además de la programación nutricional, la selección de reproductores seleccionados con alta expresión de *fads2* en células de sangre periférica permite mejorar aún más el

rendimiento de desove, larvas y juveniles, así como la capacidad de las crías para utilizar dietas con bajo contenido de FM y bajo contenido de FO.

REFERENCES

Abdelhamid, A. S., N. Martin, C. Bridges, J. S. Brainard, X. Wang, T. J. Brown, S. Hanson, O. F. Jimoh, S. M. Ajabnoor, K. H. O. Deane, F. Song and L. Hooper (2018). "Polyunsaturated fatty acids for the primary and secondary prevention of cardiovascular disease." Cochrane Database of Systematic Reviews(11).

Abi-Ayad, A., C. Mélard and P. Kestemont (1997). "Effect of n-3 fatty acids in Eurasian perch broodstock diet on egg fatty acid composition and larva stress resistance." Aquaculture International **5**: 161-168.

Acerete, L., J. C. Balasch, B. Castellana, B. Redruello, N. Roher, A. V. Canario, J. V. Planas, S. MacKenzie and L. Tort (2007). "Cloning of the glucocorticoid receptor (GR) in gilthead seabream (*Sparus aurata*): Differential expression of GR and immune genes in gilthead seabream after an immune challenge." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **148**(1): 32-43.

Adam, A.-C., K. H. Skjærven, P. Whatmore, M. Moren and K. K. Lie (2018). "Parental high dietary arachidonic acid levels modulated the hepatic transcriptome of adult zebrafish (*Danio rerio*) progeny." PLOS ONE **13**(8): e0201278.

Ainge, H., C. Thompson, S. E. Ozanne and K. B. Rooney (2010). "A systematic review on animal models of maternal high fat feeding and offspring glycaemic control." International Journal Of Obesity **35**: 325-335.

Almaida-Pagan, R., M. D. Hernandez, B. G. Garcia, J. A. Madrid, J. De Costa and P. Mendiola (2007). "Effects of total replacement of fish oil by vegetable oils on n-3 and n-6 polyunsaturated fatty acid desaturation and elongation in sharpsnout seabream (*Diplodus puntazzo*) hepatocytes and enterocytes." Aquaculture **272**(1-4): 589-598.

AOAC (1995). Official methods of analysis of AOAC International. Arlington, VA, USA, AOAC International.

Arzel, J., F. X. M. Lopez, R. Metailler, G. Stephan, M. Viau, G. Gandemer and J. Guillaume (1994). "Effect of dietary lipid on growth performance and body composition of brown trout (*Salmo trutta*) reared in seawater." Aquaculture **123**(3-4): 361-375.

Avella, M. A., G. Gioacchini, O. Decamp, P. Makridis, C. Bracciatelli and O. Carnevali (2010). "Application of multi-species of *Bacillus* in sea bream larviculture." Aquaculture **305**(1): 12-19.

Barella, L. F., P. C. F. Mathias and J. C. de Oliveira (2017). Mechanisms of programming: Pancreatic islets and fetal programming. Diet, Nutrition, and Fetal Programming. R. Rajendram, V. R. Preedy and V. B. Patel. Cham, Springer International Publishing**:** 517-528.

Basu, S. and P. Srivastava (2003). Heat shock proteins in immune response. Progress in Inflammation Research. W. van Eden. Basel, Germany, Birkhäuser**:** 33-42.

Bell, G. and W. Koppe (2010). Lipids in aquafeeds. Fish oil replacement and alternative lipid sources in aquaculture feeds. G. M. Turchini, W. K. Ng and D. Tocher. Boca Raton, FL, USA, CRC Press**:** 21-60.

Bell, J. G., A. H. McVicar, M. T. Park and J. R. Sargent (1991). "High dietary linoleic acid affects the fatty acid compositions of individual phospholipids from tissues of Atlantic salmon (*Salmo salar*): association with stress susceptibility and cardiac lesion." The Journal of Nutrition **121**(8): 1163-1172.

Bell, J. G., D. R. Tocher, B. M. Farndale, D. I. Cox, R. W. McKinney and J. R. Sargent (1997). "The effect of dietary lipid on polyunsaturated fatty acid metabolism in Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation." Lipids **32**(5): 515-525.

Bell, J. G. and R. Waagbø (2008). Safe and nutritious aquaculture produce: benefits and risks of alternative sustainable aquafeeds. Aquaculture in the ecosystem. M. Holmer, B. Kenny, C. M. Durante, N. Marbà and I. Karakassis. Luxembourg, Springer**:** 185-225.

Benatti, P., G. Peluso, R. Nicolai and M. Calvani (2004). "Polyunsaturated fatty acids: biochemical, nutritional and epigenetic properties." Journal of the American College of Nutrition **23**(4): 281-302.

Benedito-Palos, L., G. Ballester-Lozano and J. Pérez-Sánchez (2014). "Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*)." Gene **547**(1): 34-42.

Benedito-Palos, L., G. F. Ballester-Lozano, P. Simó, V. Karalazos, Á. Ortiz, J. Calduch-Giner and J. Pérez-Sánchez (2016). "Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*)." Aquaculture **454**: 8-18.

Benedito-Palos, L., A. Saera-Vila, J.-A. Calduch-Giner, S. Kaushik and J. Pérez-Sánchez (2007). "Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of GH/IGF axis." Aquaculture **267**(1-4): 199-212.

Benitez-Dorta, V., M. J. Caballero, M. Izquierdo, M. Manchado, C. Infante, M. J. Zamorano and D. Montero (2013). "Total substitution of fish oil by vegetable oils in Senegalese sole (*Solea senegalensis*) diets: effects on fish performance, biochemical composition, and expression of some glucocorticoid receptor-related genes." Fish Physiology and Biochemistry **39**(2): 335-349.

Benitez-Santana, T., R. Masuda, E. J. Carrillo, E. Ganuza, A. Valencia, C. M. Hernandez-Cruz and M. S. Izquierdo (2007). "Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae." Aquaculture **264**(1-4): 408-417.

Betancor, M. B., M. Sprague, D. Montero, S. Usher, O. Sayanova, P. J. Campbell, J. A. Napier, M. J. Caballero, M. Izquierdo and D. R. Tocher (2016). "Replacement of marine fish oil with de novo omega-3 oils from transgenic camelina sativa in feeds for gilthead sea bream (*Sparus aurata* L.)." Lipids **51**(10): 1171-1191.

Betancor, M. B., M. Sprague, O. Sayanova, S. Usher, C. Metochis, P. J. Campbell, J. A. Napier and D. R. Tocher (2016). "Nutritional evaluation of an EPA-DHA oil from transgenic

Camelina sativa in feeds for post-smolt Atlantic salmon (*Salmo salar* L.)." PLOS ONE **11**(7): e0159934.

Betancor, M. B., M. Sprague, S. Usher, O. Sayanova, P. J. Campbell, J. A. Napier and D. R. Tocher (2015). "A nutritionally-enhanced oil from transgenic *Camelina sativa* effectively replaces fish oil as a source of eicosapentaenoic acid for fish." Scientific Reports **5**: 8104.

Bolton-Smith, C., M. Woodward and R. Tavendale (1997). "Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet." European journal of clinical nutrition **51**(9): 619-624.

Boukouvala, E., M. J. Leaver, L. Favre-Krey, M. Theodoridou and G. Krey (2010). "Molecular characterization of a gilthead sea bream (*Sparus aurata*) muscle tissue cDNA for carnitine palmitoyltransferase 1B (CPT1B)." Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology **157**(2): 189-197.

Bringhenti, I., A. Schultz, T. Rachid, M. A. Bomfim, C. A. Mandarim-de-Lacerda and M. B. Aguila (2011). "An early fish oil-enriched diet reverses biochemical, liver and adipose tissue alterations in male offspring from maternal protein restriction in mice." The Journal of nutritional biochemistry **22**(11): 1009-1014.

Burdge, G. C. (2006). "Metabolism of α-linolenic acid in humans." Prostaglandins, Leukotrienes and Essential Fatty Acids **75**(3): 161-168.

Burdge, G. C. and P. C. Calder (2006). "Dietary α-linolenic acid and health-related outcomes: a metabolic perspective." Nutrition Research Reviews **19**(1): 26-52.

Burdge, G. C., M. A. Hanson, J. L. Slater-Jefferies and K. A. Lillycrop (2007). "Epigenetic regulation of transcription: A mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life?" British Journal of Nutrition **97**(6): 1036-1046.

Burdge, G. C. and K. A. Lillycrop (2010). "Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease." Annual review of nutrition **30**: 315-339.

Burton, T. and N. B. Metcalfe (2014). "Can environmental conditions experienced in early life influence future generations?" Proceedings of the Royal Society Biological Sciences Series B **281**(1785).

Caballero, M. J., A. Obach, G. Rosenlund, D. Montero, M. Gisvold and M. S. Izquierdo (2002). "Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*." Aquaculture **214**(1–4): 253-271.

Calder, P., A. Dangour, C. Diekman, A. Eilander, B. Koletzko, G. Meijer, D. Mozaffarian, H. Niinikoski, S. Osendarp and P. Pietinen (2010). "Essential fats for future health. Proceedings of the 9th Unilever Nutrition Symposium, 26–27 May 2010." European Journal of Clinical Nutrition **64**: S1-13.

Calder, P. C. (1996). "Immunomodulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids." Proceedings of the Nutrition Society **55**(2): 737-774.

Calder, P. C. (2002). "Dietary modification of inflammation with lipids." Proceedings of the Nutrition Society **61**(3): 345-358.

Calder, P. C. (2005). "Polyunsaturated fatty acids and inflammation." Biochemical Society Transactions **33**(2): 423-427.

Calder, P. C. (2006). "n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases." American Journal of Clinical Nutrition **83**.

Calder, P. C. (2018). "Very long-chain n-3 fatty acids and human health: fact, fiction and the future." Proceedings of the Nutrition Society **77**(1): 52-72.

Cashion, T., F. Le Manach, D. Zeller and D. Pauly (2017). "Most fish destined for fishmeal production are food-grade fish." Fish and Fisheries **18**(5): 837-844.

Cassar-Malek, I., B. Picard, C. Bernard and J.-F. Hocquette (2008). "Application of gene expression studies in livestock production systems: a European perspective." Australian Journal of Experimental Agriculture **48**(7): 701-710.

Castro, L. F., D. R. Tocher and O. Monroig (2016). "Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire." Progress in Lipid Research **62**: 25-40.

Cetin, I. and B. Koletzko (2008). "Long-chain ω-3 fatty acid supply in pregnancy and lactation." Current Opinion in Clinical Nutrition & Metabolic Care **11**(3): 297-302.

Childs, C. E., S. P. Hoile, G. C. Burdge and P. C. Calder (2012). "Changes in rat n-3 and n-6 fatty acid composition during pregnancy are associated with progesterone concentrations and hepatic FADS2 expression." Prostaglandins, Leukotrienes and Essential Fatty Acids **86**(4): 141-147.

Christie, W. W. (1982). Lipid analysis. Oxford, UK, Pergamon Press.

Clarke, S. D. (2001). "Polyunsaturated Fatty Acid Regulation of Gene Transcription: A Molecular Mechanism to Improve the Metabolic Syndrome." Journal of Nutrition **131**(4): 1129-1132.

Clarkson, M., H. Migaud, C. Metochis, L. M. Vera, D. Leeming, D. R. Tocher and J. F. Taylor (2017). "Early nutritional intervention can improve utilisation of vegetable-based diets in diploid and triploid Atlantic salmon (*Salmo salar* L.)." British Journal of Nutrition: 1-13.

Colhoun, E. H. and M. V. Smith (1960). "Neurohormonal properties of royal jelly." Nature **188**: 854-855.

Connor, W. E. (2000). "Importance of n–3 fatty acids in health and disease." The American Journal of Clinical Nutrition **71**(1): 171S-175S.

Corbin, K. D. (2011). Metabolic Syndrome, Obesity, and Diabetes. Nutrition in Epigenetics. M. D. Niculescu and P. Haggarty. Oxford, UK, Wiley-Blackwell**:** 145-164.

Council, N. R. (2015). Critical role of animal science research in food security and sustainability, National Academies Press.

Cuesta, A., M. Ángeles Esteban and J. Meseguer (2006). "Cloning, distribution and up-regulation of the teleost fish MHC class II alpha suggests a role for granulocytes as antigen-presenting cells." Molecular Immunology **43**(8): 1275-1285.

Curley, J. P., R. Mashoodh and F. A. Champagne (2011). "Epigenetics and the origins of paternal effects." Hormones and Behavior **59**(3): 306-314.

De Silva, S., D. S. Francis and A. G. Tacon (2011). Fish oil in aquaculture: in retrospect. Fish oil replacement and alternative lipid sources in aquaculture feeds. G. M. Turchini, W. K. Ng and D. Tocher. Boca Raton, Florida, CRC Press**:** 1-20.

Domenichiello, A. F., A. P. Kitson and R. P. Bazinet (2015). "Is docosahexaenoic acid synthesis from α-linolenic acid sufficient to supply the adult brain?" Progress in Lipid Research **59**: 54-66.

Duque-Guimaraes, D. E. and S. E. Ozanne (2013). "Nutritional programming of insulin resistance: causes and consequences." Trends in Endocrinology and Metabolism **24**(10): 525-535.

Duray, M., H. Kohno and F. Pascual (1994). "The effect of lipid-enriched broodstock diets on spawning and on egg and larval quality of hatchery-bred rabbitfish (*Siganus guttatus*)." The Philippine Scientist(31): 42-57.

Engrola, S., C. Aragão, L. M. Valente and L. E. Conceição (2018). Nutritional Modulation of Marine Fish Larvae Performance. Emerging Issues in Fish Larvae Research. M. Yúfera. Cham, Switzerland, Springer**:** 209-228.

Eroldoğan, T. O., A. H. Yılmaz, G. M. Turchini, M. Arslan, N. A. Sirkecioğlu, K. Engin, I. Özşahinoğlu and P. Mumoğullarında (2013). "Fatty acid metabolism in European sea bass (*Dicentrarchus labrax*): effects of n-6 PUFA and MUFA in fish oil replaced diets." Fish Physiology and Biochemistry **39**(4): 941-955.

Eryalçın, K. M., J. Roo, R. Saleh, E. Atalah, T. Benítez, M. Betancor, M. del Carmen Hernandez-Cruz and M. Izquierdo (2013). "Fish oil replacement by different microalgal products in microdiets for early weaning of gilthead sea bream (*Sparus aurata*, L.)." Aquaculture Research **44**(5): 819-828.

Fang, L., X. F. Liang, Y. Zhou, X. Z. Guo, Y. He, T. L. Yi, L. W. Liu, X. C. Yuan and Y. X. Tao (2014). "Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, *Danio rerio*." British Journal of Nutrition **111**(5): 808-818.

FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Roma, Italy, FAO**:** 200.

FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome, Italy, Food & Agriculture Org.

FAO (2018). "The State of World Fisheries and Aquaculture 2018—Meeting the Sustainable Development Goals."

Farooqui, A. A., L. A. Horrocks and T. Farooqui (2007). "Modulation of inflammation in brain: a matter of fat." Journal of Neurochemistry **101**(3): 577-599.

Feil, R. (2006). "Environmental and nutritional effects on the epigenetic regulation of genes." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **600**(1): 46-57.

Fernández-Palacios, H. (2005). "Efecto de distintas dietas para reproductores de Doradas (*Sparus aurata*) sobre la calidad de sus puestas."

Fernández-Palacios, H., C. Hernández, J. Fernández-Palacios, J. Vergara and L. Robaina (1990). Influencia de distintas proporciones hembra: macho en la puesta de dorada (*Sparus aurata* L.). Proceedings of the III National Congress on Aquaculture.

Fernández-Palacios, H., M. Izquierdo, L. Robaina, A. Valencia, M. Salhi and D. Montero (1997). "The effect of dietary protein and lipid from squid and fish meals on egg quality of broodstock for gilthead seabream (Sparus aurata)." Aquaculture **148**(2): 233-246.

Fernández-Palacios, H., M. S. Izquierdo, L. Robaina, A. Valencia, M. Salhi and J. Vergara (1995). "Effect of *n*–3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L.)." Aquaculture **132**(3): 325-337.

Fernández-Palacios, H., B. Norberg, M. Izquierdo and K. Hamre (2011). Effects of broodstock diet on eggs and larvae. Larval Fish Nutrition. J. G. Holt. Oxford, UK, Wiley-Blackwell**:** 151-181.

Folch, J., M. Lees and G. Sloane-Stanley (1957). "A simple method for the isolation and purification of total lipids from animal tissues." Journal of Biological Chemistry **226**(1): 497-509.

Fontagné-Dicharry, S., H. Alami-Durante, C. Aragão, S. J. Kaushik and I. Geurden (2017). "Parental and early-feeding effects of dietary methionine in rainbow trout (Oncorhynchus mykiss)." Aquaculture **469**: 16-27.

Foundations, B. N. (1994). Unsaturated fatty acids: nutritional and physiological significance; the report of the British Nutrition Foundations Task Force, London: Chapman & Hall.

Francis, D. S., D. J. Peters and G. M. Turchini (2009). "Apparent in Vivo Δ-6 Desaturase Activity, Efficiency, and Affinity Are Affected by Total Dietary C18 PUFA in the Freshwater Fish Murray Cod." Journal of Agricultural and Food Chemistry **57**(10): 4381-4390.

Fuiman, L. A. and K. O. Perez (2015). "Metabolic programming mediated by an essential fatty acid alters body composition and survival skills of a marine fish." Proceedings of the Royal Society of London B: Biological Sciences **282**(1819).

Ganga, R. (2010). Effect of feeding gilthead seabream (*Sparus aurata*) with different levels of n-3 and n-6 lipids vegetable oils on fish health and resistance to stress, Universidad de Las Palmas de Gran Canaria.

Ganga, R., J. G. Bell, D. Montero, L. Robaina, M. J. Caballero and M. S. Izquierdo (2005). "Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*)." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **142**(4): 410-418.

Ganga, R., L. Tort, L. Acerete, D. Montero and M. Izquierdo (2006). "Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*." Journal of endocrinology **190**(1): 39-45.

García-Castillo, J., P. Pelegrín, V. Mulero and J. Meseguer (2002). "Molecular cloning and expression analysis of tumor necrosis factor α from a marine fish reveal its constitutive expression and ubiquitous nature." Immunogenetics **54**(3): 200-207.

Gatlin, D. M., F. T. Barrows, P. Brown, K. Dabrowski, T. G. Gaylord, R. W. Hardy, E. Herman, G. S. Hu, A. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E. J. Souza, D. Stone, R. Wilson and E. Wurtele (2007). "Expanding the utilization of sustainable plant products in aquafeeds: a review." Aquaculture Research **38**(6): 551-579.

Geay, F., I. C. E. Santigosa, C. Corporeau, P. Boudry, Y. Dreano, L. Corcos, N. Bodin, M. Vandeputte, J. L. Zambonino-Infante, D. Mazurais and C. L. Cahu (2010). "Regulation of FADS2 expression and activity in European sea bass (*Dicentrarchus labrax*, L.) fed a vegetable diet." Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology **156**(4): 237-243.

Geurden, I., M. Aramendi, J. Zambonino-Infante and S. Panserat (2007). "Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **292**(6): R2275-R2283.

Geurden, I., P. Borchert, M. N. Balasubramanian, J. W. Schrama, M. Dupont-Nivet, E. Quillet, S. J. Kaushik, S. Panserat and F. Médale (2013). "The Positive Impact of the Early-Feeding of a Plant-Based Diet on Its Future Acceptance and Utilisation in Rainbow Trout." PloS one **8**(12): e83162.

Gibson, R. A., M. A. Neumann, E. L. Lien, K. A. Boyd and W. C. Tu (2013). "Docosahexaenoic acid synthesis from alpha-linolenic acid is inhibited by diets high in polyunsaturated fatty acids." Prostaglandins, Leukotrienes and Essential Fatty Acids **88**(1): 139-146.

Glencross, B., W. Hawkins and J. Curnow (2003). "Evaluation of canola oils as alternative lipid resources in diets for juvenile red seabream, *Pagrus auratus*." Aquaculture Nutrition **9**(5): 305-315.

Glencross, B. D. (2009). "Exploring the nutritional demand for essential fatty acids by aquaculture species." Reviews in Aquaculture **1**(2): 71-124.

Glencross, B. D., W. E. Hawkins and J. G. Curnow (2003). "Restoration of the fatty acid composition of red seabream (*Pagrus auratus*) using a fish oil finishing diet after grow-out on plant oil based diets." Aquaculture Nutrition **9**(6): 409-418.

Gluckman, P. D. and M. A. Hanson (2004). "Living with the Past: Evolution, Development, and Patterns of Disease." Science **305**(5691): 1733-1736.

Gluckman, P. D., M. A. Hanson, C. Cooper and K. L. Thornburg (2008). "Effect of In Utero and Early-Life Conditions on Adult Health and Disease." New England Journal of Medicine **359**(1): 61-73.

Gluckman, P. D., M. A. Hanson and H. G. Spencer (2005). "Predictive adaptive responses and human evolution." Trends in Ecology & Evolution **20**(10): 527-533.

Gluckman, P. D., M. A. Hanson, H. G. Spencer and P. Bateson (2005). "Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies." Proceedings of the Royal Society B: Biological Sciences **272**(1564): 671-677.

Gotoh, T. (2015). "Potential of the application of epigenetics in animal production." Animal Production Science **55**(2): 145-158.

Govoni, J. J., G. W. Boehlert and Y. Watanabe (1986). The physiology of digestion in fish larvae. Contemporary studies on fish feeding: the proceedings of GUTSHOP'84, Springer**:** 59-78.

Hales, C. N. and D. J. Barker (2001). "The thrifty phenotype hypothesis." Br Med Bull **60**: 5-20.

Hamoutene, D., L. Lush, D. Drover and A. Walsh (2009). "Investigation of the temporal effects of spawning season and maternal and paternal differences on egg quality in Atlantic cod *Gadus morhua* L. broodstock." Aquaculture Research **40**(14): 1668-1669.

Hardy, R. W. (2010). "Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal." Aquaculture Research **41**(5): 770-776.

Hastings, N., M. Agaba, D. R. Tocher, M. J. Leaver, J. R. Dick, J. R. Sargent and A. J. Teale (2001). "A vertebrate fatty acid desaturase with Δ5 and Δ6 activities." Proceedings of the National Academy of Sciences **98**(25): 14304-14309.

Health, U. D. o. and H. Services (2015). "2015–2020 Dietary guidelines for Americans." Washington (DC): USDA.

Hoile, S. P., N. A. Irvine, C. J. Kelsall, C. Sibbons, A. Feunteun, A. Collister, C. Torrens, P. C. Calder, M. A. Hanson, K. A. Lillycrop and G. C. Burdge (2013). "Maternal fat intake in rats alters 20:4n-6 and 22:6n-3 status and the epigenetic regulation of Fads2 in offspring liver." J Nutr Biochem **24**(7): 1213-1220.

Hwang, S. W., H. Cho, J. Kwak, S. Y. Lee, C. J. Kang, J. Jung, S. Cho, K. H. Min, Y. G. Suh, D. Kim and U. Oh (2000). "Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances." Proc Natl Acad Sci U S A **97**(11): 6155-6160.

Hyatt, M. A., G. S. Gopalakrishnan, J. Bispham, S. Gentili, I. C. McMillen, S. M. Rhind, M. T. Rae, C. E. Kyle, A. N. Brooks, C. Jones, H. Budge, D. Walker, T. Stephenson and M. E. Symonds (2007). "Maternal nutrient restriction in early pregnancy programs hepatic mRNA expression of growth-related genes and liver size in adult male sheep." Journal of Endocrinology **192**(1): 87-97.

Ibeas, C., M. S. Izquierdo and A. Lorenzo (1994). "Effect of different levels of n–3 highly unsaturated fatty acids on growth and fatty acid composition of juvenile gilthead seabream (*Sparus aurata*)." Aquaculture **127**(2): 177-188.

Ishikawa, T. O. and H. R. Herschman (2007). "Two inducible, functional cyclooxygenase-2 genes are present in the rainbow trout genome." Journal of Cellular Biochemistry **102**(6): 1486-1492.

Izquierdo, M. (2005). "Essential fatty acid requirements in Mediterranean fish species." Cah. Options Mediterr **63**: 91-102.

Izquierdo, M. (2013). How relevant is hatchery nutrition for the long-term performance of fish in the farm? Keynote Speech. Research for the Next 40 Years of Sustainable Global Aquaculture. B. Costa-Pierce, B. Austin, J. Benzie, A. Farrell, D. Gatlin, G. Hulata and D. Little. Las Palmas, Spain, Elsevier.

Izquierdo, M. (2013). Nutritional programming and progeny performance in marine fish. EPICONCEPT Workshop 2013 Epigenetics for Improved Food Production: from Model to Practice, Sant Feliu de Guíxols, Spain, Societat Catalana de Biologia (Catalan Society of Biology) and the COST Action FA1201.

Izquierdo, M., M. Scolamacchia, M. Betancor, J. Roo, M. Caballero, G. Terova and P. Witten (2013). "Effects of dietary DHA and α-tocopherol on bone development, early mineralisation and oxidative stress in *Sparus aurata* (Linnaeus, 1758) larvae." British Journal of Nutrition **109**(10): 1796-1805.

Izquierdo, M., J. Socorro, L. Arantzamendi and C. Hernández-Cruz (2000). "Recent advances in lipid nutrition in fish larvae." Fish Physiology and Biochemistry **22**(2): 97-107.

Izquierdo, M. S. (1996). "Essential fatty acid requirements of cultured marine fish larvae." Aquaculture Nutrition **2**(4): 183-191.

Izquierdo, M. S., H. Fernandez-Palacios and A. G. J. Tacon (2001). "Effect of broodstock nutrition on reproductive performance of fish." Aquaculture **197**(1): 25-42.

Izquierdo, M. S. and W. Koven (2011). Lipids. Larval Fish Nutrition. J. G. Holt, Wiley-Blackwell**:** 47-81.

Izquierdo, M. S., D. Montero, L. Robaina, M. J. Caballero, G. Rosenlund and R. Ginés (2005). "Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding." Aquaculture **250**(1-2): 431-444.

Izquierdo, M. S., A. Obach, L. Arantzamendi, D. Montero, L. Robaina and G. Rosenlund (2003). "Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality." Aquaculture Nutrition **9**(6): 397-407.

Izquierdo, M. S., L. Robaina, E. Juárez-Carrillo, V. Oliva, C. Hernández-Cruz and J. Afonso (2008). "Regulation of growth, fatty acid composition and delta 6 desaturase expression by dietary lipids in gilthead seabream larvae (*Sparus aurata*)." Fish Physiology and Biochemistry **34**(2): 117-127.

Izquierdo, M. S., S. Turkmen, D. Montero, M. J. Zamorano, J. M. Afonso, V. Karalazos and H. Fernández-Palacios (2015). "Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream." Aquaculture **449**: 18-26.

Izquierdo, M. S., T. Watanabe, T. Takeuchi, T. Arakawa and C. Kitajima (1990). Optimum EFA levels in Artemia to meet the EFA requirements of red sea bream (*Pagrus major*). The Current Status of Fish Nutrition in Aquaculture: The Proceedings of the Third International Symposium on Feeding and Nutrition in Fish. Toba, Japan**:** 221-232.

Jaenisch, R. and A. Bird (2003). "Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals." Nature Genetics **33**: 245-254.

Janssen, K., H. Chavanne, P. Berentsen and H. Komen (2017). "Impact of selective breeding on European aquaculture." Aquaculture **472**: 8-16.

Jordal, A. E., B. E. Torstensen, S. Tsoi, D. R. Tocher, S. P. Lall and S. E. Douglas (2005). "Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.)." Journal of Nutrition **135**.

Juaneda, P. and G. Rocquelin (1985). "Rapid and convenient separation of phospholipids and non phosphorus lipids from rat heart using silica cartridges." Lipids **20**(1): 40-41.

Kadmon, G., Z. Yaron and H. Gordin (1985). "Sequence of gonadal events and oestradiol levels in Sparus aurata (L.) under two photoperiod regimes." Journal of Fish Biology **26**(5): 609-620.

Kaushik, S., D. Coves, G. Dutto and D. Blanc (2004). "Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*." Aquaculture **230**(1): 391-404.

Kaushik, S. and M. Troell (2010). "Taking the fish-in fish-out ratio a step further." Aquaculture Europe **35**(1): 15-17.

Kelsall, C. J., S. P. Hoile, N. A. Irvine, M. Masoodi, C. Torrens, K. A. Lillycrop, P. C. Calder, G. F. Clough, M. A. Hanson and G. C. Burdge (2012). "Vascular Dysfunction Induced in Offspring by Maternal Dietary Fat Involves Altered Arterial Polyunsaturated Fatty Acid Biosynthesis." PLOS ONE **7**(4): e34492.

Kjaer, M. A., M. Todorcevic, B. E. Torstensen, A. Vegusdal and B. Ruyter (2008). "Dietary n-3 HUFA affects mitochondrial fatty acid beta-oxidation capacity and susceptibility to oxidative stress in Atlantic salmon." Lipids **43**(9): 813-827.

Kolditz, C., M. Borthaire, N. Richard, G. Corraze, S. Panserat, C. Vachot, F. Lefèvre and F. Médale (2008). "Liver and muscle metabolic changes induced by dietary energy content and genetic selection in rainbow trout (*Oncorhynchus mykiss*)." American Journal of Physiology: Regulatory, Integrative and Comparative Physiology **294**(4): R1154-1164.

Koletzko, B., E. Lattka, S. Zeilinger, T. Illig and C. Steer (2010). "Genetic variants of the fatty acid desaturase gene cluster predict amounts of red blood cell docosahexaenoic and other polyunsaturated fatty acids in pregnant women: findings from the Avon Longitudinal Study of Parents and Children–." The American journal of clinical nutrition **93**(1): 211-219.

Koven, W., Y. Barr, S. Lutzky, I. Ben-Atia, R. Weiss, M. Harel, P. Behrens and A. Tandler (2001). "The effect of dietary arachidonic acid (20:4n–6) on growth, survival and resistance to handling stress in gilthead seabream (Sparus aurata) larvae." Aquaculture **193**(1): 107-122.

Kremmyda, L.-S., M. Vlachava, P. S. Noakes, N. D. Diaper, E. A. Miles and P. C. Calder (2011). "Atopy risk in infants and children in relation to early exposure to fish, oily fish, or long-chain omega-3 fatty acids: a systematic review." Clinical reviews in allergy & immunology **41**(1): 36-66.

Kucharski, R., J. Maleszka, S. Foret and R. Maleszka (2008). "Nutritional control of reproductive status in honeybees via DNA methylation." Science **319**(5871): 1827-1830.

Kumar, K. A., A. Lalitha, D. Pavithra, I. J. Padmavathi, M. Ganeshan, K. R. Rao, L. Venu, N. Balakrishna, N. H. Shanker and S. U. Reddy (2013). "Maternal dietary folate and/or vitamin B12 restrictions alter body composition (adiposity) and lipid metabolism in Wistar rat offspring." The Journal of nutritional biochemistry **24**(1): 25-31.

Laizé, V., A. R. Pombinho and M. L. Cancela (2005). "Characterization of *Sparus aurata* osteonectin cDNA and in silico analysis of protein conserved features: Evidence for more than one osteonectin in Salmonidae." Biochimie **87**(5): 411-420.

Langley-Evans, S. C. (2015). "Nutrition in early life and the programming of adult disease: a review." Journal of Human Nutrition and Dietetics **28**: 1-14.

Le Boucher, R., M. Dupont-Nivet, M. Vandeputte, T. Kerneïs, L. Goardon, L. Labbé, B. Chatain, M. J. Bothaire, L. Larroquet, F. Médale and E. Quillet (2012). "Selection for Adaptation to Dietary Shifts: Towards Sustainable Breeding of Carnivorous Fish." PLoS ONE **7**(9): e44898.

Le Boucher, R., M. Vandeputte, M. Dupont-Nivet, E. Quillet, F. Ruelle, A. Vergnet, S. Kaushik, J. M. Allamellou, F. Medale and B. Chatain (2013). "Genotype by diet interactions in European sea bass (*Dicentrarchus labrax* L. 1756): nutritional challenge with totally plant-based diets." Journal of Animal Science **91**(1): 44-56.

Le Boucher, R., M. Vandeputte, M. Dupont-Nivet, E. Quillet, D. Mazurais, J. Robin, A. Vergnet, F. Medale, S. Kaushik and B. Chatain (2011). "A first insight into genotype x diet interactions in European sea bass (*Dicentrarchus labrax* L. 1756) in the context of plant-based diet use." Aquaculture Research **42**(4): 583-592.

Leaver, M. J., J. M. Bautista, B. T. Bjornsson, E. Jonsson, G. Krey, D. R. Tocher and B. E. Torstensen (2008). "Towards fish lipid nutrigenomics: Current state and prospects for fin-fish aquaculture." Reviews in Fisheries Science **16**: 73-94.

Leaver, M. J., L. A. Villeneuve, A. Obach, L. Jensen, J. E. Bron, D. R. Tocher and J. B. Taggart (2008). "Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*)." BMC Genomics **9**: 299.

Li, S., K. Mai, W. Xu, Y. Yuan, Y. Zhang and Q. Ai (2014). "Characterization, mRNA expression and regulation of Δ6 fatty acyl desaturase (FADS2) by dietary n-3 long chain polyunsaturated fatty acid (LC-PUFA) levels in grouper larvae (*Epinephelus coioides*)." Aquaculture **434**(0): 212-219.

Lillycrop, K. A. and G. C. Burdge (2018). Chapter 13 - Interactions Between Polyunsaturated Fatty Acids and the Epigenome. Polyunsaturated Fatty Acid Metabolism, AOCS Press**:** 225-239.

Lillycrop, K. A., E. S. Phillips, A. A. Jackson, M. A. Hanson and G. C. Burdge (2005). "Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring." Journal of Nutrition **135**(6): 1382-1386.

Lillycrop, K. A., E. S. Phillips, C. Torrens, M. A. Hanson, A. A. Jackson and G. C. Burdge (2008). "Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPARα promoter of the offspring." British Journal of Nutrition **100**(02): 278-282.

Lin, H. Z., Y. J. Liu, J. G. He, W. H. Zheng and L. X. Tian (2007). "Alternative vegetable lipid sources in diets for grouper, *Epinephelus coioides* (Hamilton): effects on growth, and muscle and liver fatty acid composition." Aquaculture Research **38**(15): 1605-1611.

Liu, J., M. J. Caballero, Eacute, M. Izquierdo, T. E.-S. Ali, Hern, Aacute, C. M. Ndez-Cruz, Iacute, A. Valencia, Fern, Aacute, H. I. P. Ndez-Palacios, Oacute and Lito (2002). "Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream *Sparus aurata*." Fisheries science **68**(6): 1165-1172.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method." Methods **25**.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lucas, A. (1998). "Programming by Early Nutrition: An Experimental Approach." The Journal of Nutrition **128**(2): 401-406.

Luiting, P. (1990). "Genetic variation of energy partitioning in laying hens: causes of variation in residual feed consumption." World's Poultry Science Journal **46**(2): 133-152.

Lupu, D. S., C. L. Cheatham, K. D. Corbin and M. D. Niculescu (2015). "Genetic and epigenetic transgenerational implications related to omega-3 fatty acids. Part I: maternal FADS2 genotype and DNA methylation correlate with polyunsaturated fatty acid status in toddlers: an exploratory analysis." Nutrition Research **35**(11): 939-947.

Marandel, L., V. Véron, A. Surget, É. Plagnes-Juan and S. Panserat (2016). "Glucose metabolism ontogenesis in rainbow trout (Oncorhynchus mykiss) in the light of the recently sequenced genome: new tools for intermediary metabolism programming." Journal of Experimental Biology: jeb. 134304.

Marcheselli, V. L., S. Hong, W. J. Lukiw, X. H. Tian, K. Gronert, A. Musto, M. Hardy, J. M. Gimenez, N. Chiang, C. N. Serhan and N. G. Bazan (2003). "Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression." J Biol Chem **278**(44): 43807-43817.

Mathers, J. (2007). Early nutrition: impact on epigenetics. Nutrigenomics-Opportunities in Asia, Southeast Asia Region, Singapore, Karger Publishers.

Matsuzaka, T. and H. Shimano (2009). "Elovl6: a new player in fatty acid metabolism and insulin sensitivity." Journal of Molecular Medicine **87**(4): 379-384.

Matsuzaka, T., H. Shimano, N. Yahagi, T. Kato, A. Atsumi, T. Yamamoto, N. Inoue, M. Ishikawa, S. Okada, N. Ishigaki, H. Iwasaki, Y. Iwasaki, T. Karasawa, S. Kumadaki, T. Matsui, M. Sekiya, K. Ohashi, A. H. Hasty, Y. Nakagawa, A. Takahashi, H. Suzuki, S. Yatoh, H. Sone, H. Toyoshima, J.-i. Osuga and N. Yamada (2007). "Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance." Nature Medicine **13**: 1193.

Mellery, J., J. Brel, J. Dort, F. Geay, P. Kestemont, D. S. Francis, Y. Larondelle and X. Rollin (2017). "A n-3 PUFA depletion applied to rainbow trout fry (*Oncorhynchus mykiss*) does not modulate its subsequent lipid bioconversion capacity." British Journal of Nutrition **117**(2): 187-199.

Michl, S. (2017). Evaluation of plastic responses to nutritional programming by various feed sources in brown and rainbow trout fry, Christian-Albrechts-Universität zu Kiel.

Monaghan, P. (2008). "Early growth conditions, phenotypic development and environmental change." Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences **363**(1497): 1635-1645.

Monroig, Ó., J. C. Navarro and D. R. Tocher (2011). "Long-chain polyunsaturated fatty acids in fish: recent advances on desaturases and elongases involved in their biosyntesis." Avances en Nutrición Acuícola **11**: 257-283.

Monroig, O., D. R. Tocher and L. F. C. Castro (2018). Chapter 3 - Polyunsaturated Fatty Acid Biosynthesis and Metabolism in Fish A2 - Burdge, Graham C. Polyunsaturated Fatty Acid Metabolism, AOCS Press**:** 31-60.

Monroig, Ó., D. R. Tocher and J. C. Navarro (2013). "Biosynthesis of Polyunsaturated Fatty Acids in Marine Invertebrates: Recent Advances in Molecular Mechanisms." Marine Drugs **11**(10): 3998-4018.

Monroig, Ó., S. Wang, L. Zhang, C. You, D. R. Tocher and Y. Li (2012). "Elongation of long-chain fatty acids in rabbitfish *Siganus canaliculatus*: Cloning, functional characterisation and tissue distribution of Elovl5- and Elovl4-like elongases." Aquaculture **350-353**(0): 63-70.

Montero, D., V. Grasso, M. S. Izquierdo, R. Ganga, F. Real, L. Tort, M. J. Caballero and F. Acosta (2008). "Total substitution of fish oil by vegetable oils in gilthead sea bream (*Sparus aurata*) diets: Effects on hepatic Mx expression and some immune parameters." Fish & Shellfish Immunology **24**(2): 147-155.

Montero, D. and M. Izquierdo (2010). Welfare and health of fish fed vegetable oils as alternative lipid sources to fish oil. Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. G. M. Turchini, W. K. Ng and D. R. Tocher. Boca Raton, Florida, USA, CRC Press**:** 439-485.

Montero, D., L. Robaina, M. J. Caballero, R. Ginés and M. S. Izquierdo (2005). "Growth, feed utilization and flesh quality of European sea bass (*Dicentrarchus labrax*) fed diets containing vegetable oils: A time-course study on the effect of a re-feeding period with a 100% fish oil diet." Aquaculture **248**(1–4): 121-134.

Montero, D., G. Terova, S. Rimoldi, L. Tort, D. Negrin, M. J. Zamorano and M. Izquierdo (2015). "Modulation of adrenocorticotrophin hormone (ACTH)-induced expression of stress-related genes by PUFA in inter-renal cells from European sea bass (*Dicentrarchus labrax*)." Journal of Nutritional Science **4**: e16.

Montero, D., L. Tort, M. Izquierdo, J. Socorro, L. Robaina, J. Vergara and H. Fernández-Palacios (1996). Effect of α-tocopherol and n-3 HUFA deficient diets on blood cells, selected immune parameters and proximate body composition of gilthead seabream (*Sparus aurata*). Modulators of Immune Response The Evolutionary Trail. J. S. Stolen, T. C.

Fletcher, C. J. Secombes, J. L. Zelikoff, L. Twerdock and D. P. Anderson. Fair Haven, CT, USA, SOS Publications. **9:** 251-266.

Montero, D., L. Tort, M. S. Izquierdo, L. Robaina and J. M. Vergara (1998). "Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies." Fish Physiology and Biochemistry **18**(4): 399-407.

Moon, Y.-A., C. R. Ochoa, M. A. Mitsche, R. E. Hammer and J. D. Horton (2014). "Deletion of ELOVL6 blocks the synthesis of oleic acid but does not prevent the development of fatty liver or insulin resistance." Journal of Lipid Research **55**(12): 2597-2605.

Morais, S., A. C. Mendes, M. F. Castanheira, J. Coutinho, N. Bandarra, J. Dias, L. E. C. Conceição and P. Pousão-Ferreira (2014). "New formulated diets for *Solea senegalensis* broodstock: Effects of parental nutrition on biosynthesis of long-chain polyunsaturated fatty acids and performance of early larval stages and juvenile fish." Aquaculture **432**: 374-382.

Morash, A. J., D. P. Bureau and G. B. McClelland (2009). "Effects of dietary fatty acid composition on the regulation of carnitine palmitoyltransferase (CPT) I in rainbow trout (*Oncorhynchus mykiss*)." Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology **152**(1): 85-93.

Morgan, H. D., H. G. Sutherland, D. I. Martin and E. Whitelaw (1999). "Epigenetic inheritance at the agouti locus in the mouse." Nature Genetics **23**(3): 314-318.

Mossa, F., S. W. Walsh, J. J. Ireland and A. C. O. Evans (2015). "Early nutritional programming and progeny performance: Is reproductive success already set at birth?" Animal Frontiers **5**(1): 18-24.

Mourente, G. and J. M. Odriozola (1990). "Effect of broodstock diets on total lipids and fatty acid composition of larvae of gilthead sea bream (*Sparus aurata* L.) during yolksac stage." Fish Physiology and Biochemistry **8**(2): 103-110.

Mozaffarian, D. and J. H. Y. Wu (2012). "(n-3) Fatty Acids and Cardiovascular Health: Are Effects of EPA and DHA Shared or Complementary?" The Journal of Nutrition **142**(3): 614S-625S.

Navarro-Guillén, C., S. Engrola, F. Castanheira, N. Bandarra, I. Hachero-Cruzado, D. R. Tocher, L. E. C. Conceição and S. Morais (2014). "Effect of varying dietary levels of LC-PUFA and vegetable oil sources on performance and fatty acids of Senegalese sole post larvae: Puzzling results suggest complete biosynthesis pathway from C18 PUFA to DHA." Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology **167**(0): 51-58.

Naylor, R. L., R. W. Hardy, D. P. Bureau, A. Chiu, M. Elliott, A. P. Farrell, I. Forster, D. M. Gatlin, R. J. Goldburg, K. Hua and P. D. Nichols (2009). "Feeding aquaculture in an era of finite resources." Proceedings of the National Academy of Sciences of the United States of America **106**(36): 15103-15110.

Ng, W.-K. and V. Gibon (2010). "Palm oil and saturated fatty acid-rich vegetable oils." Fish oil replacement and alternative lipid sources in aquaculture feeds: 99-132.

Orsavova, J., L. Misurcova, J. V. Ambrozova, R. Vicha and J. Mlcek (2015). "Fatty Acids Composition of Vegetable Oils and Its Contribution to Dietary Energy Intake and

Dependence of Cardiovascular Mortality on Dietary Intake of Fatty Acids." International journal of molecular sciences **16**(6): 12871-12890.

Öst, A. and J. A. Pospisilik (2015). "Epigenetic modulation of metabolic decisions." Current Opinion in Cell Biology **33**: 88-94.

Otero-Ferrer, F., M. Izquierdo, A. Fazeli and W. V. Holt (2016). "Embryonic developmental plasticity in the long-snouted seahorse (*Hippocampus reidi*, Ginsburg 1933) in relation to parental preconception diet." Reproduction, Fertility and Development **28**(7): 1020-1028.

Otero-Ferrer, F., M. Izquierdo, A. Segade, A. Fazeli and W. Holt (2013). Seahorses: A new and unique epigenetic model. EPICONCEPT Workshop 2013 Epigenetics for Improved Food Production: from Model to Practice, Sant Feliu de Guíxols, Spain.

Panserat, S., L. Marandel, I. Geurden, V. Veron, K. Dias, E. Plagnes-Juan, G. Pegourié, E. Arbenoits, E. Santigosa, G. Weber and V. Verlhac Trichet (2017). "Muscle catabolic capacities and global hepatic epigenome are modified in juvenile rainbow trout fed different vitamin levels at first feeding." Aquaculture **468**: 515-523.

Panserat, S., L. Marandel, I. Seiliez and S. Skiba-Cassy (2019). "New Insights on Intermediary Metabolism for a Better Understanding of Nutrition in Teleosts." Annual Review of Animal Biosciences **7**(1): null.

Pauletto, M., T. Manousaki, S. Ferraresso, M. Babbucci, A. Tsakogiannis, B. Louro, N. Vitulo, V. H. Quoc, R. Carraro and D. Bertotto (2018). "Genomic analysis of Sparus aurata reveals the evolutionary dynamics of sex-biased genes in a sequential hermaphrodite fish." Communications biology **1**(1): 119.

Pelegrín, P., J. García-Castillo, V. Mulero and J. Meseguer (2001). "Interleukin-1β isolated from a marine fish reveals up-regulated expression in macrophages following activation with lipopolysaccharide and lymphokines." Cytokine **16**(2): 67-72.

Perez, K. O. and L. A. Fuiman (2015). "Maternal diet and larval diet influence survival skills of larval red drum Sciaenops ocellatus." Journal of Fish Biology **86**(4): 1286-1304.

Petursdottir, D. H. and I. Hardardottir (2007). "Dietary fish oil increases the number of splenic macrophages secreting TNF-alpha and IL-10 but decreases the secretion of these cytokines by splenic T cells from mice." J Nutr **137**(3): 665-670.

Piedecausa, M. A., M. J. Mazon, B. G. Garcia and M. D. Hernandez (2007). "Effects of total replacement of fish oil by vegetable oils in the diets of sharpsnout seabream (*Diplodus puntazzo*)." Aquaculture **263**(1-4): 211-219.

Raclot, T., R. Groscolas, D. Langin and P. Ferre (1997). "Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues." Journal of Lipid Research **38**(10): 1963-1972.

Reddy, K. S. (2002). "Cardiovascular diseases in the developing countries: dimensions, determinants, dynamics and directions for public health action." Public Health Nutr **5**(1a): 231-237.

Reynolds, L. P. and J. S. Caton (2012). "Role of the pre-and post-natal environment in developmental programming of health and productivity." Molecular and cellular endocrinology **354**(1): 54-59.

Robaina, L., M. Izquierdo, F. Moyano, J. Socorro, J. Vergara, D. Montero and H. Fernandez-Palacios (1995). "Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications." Aquaculture **130**(2-3): 219-233.

Robaina, L., M. S. Izquierdo, F. J. Moyano, J. Socorro, J. M. Vergara and D. Montero (1998). "Increase of the dietary n–3/n–6 fatty acid ratio and addition of phosphorus improves liver histological alterations induced by feeding diets containing soybean meal to gilthead seabream, Sparus aurata." Aquaculture **161**(1): 281-293.

Rocha, F., J. Dias, S. Engrola, P. Gavaia, I. Geurden, M. T. Dinis and S. Panserat (2014). "Glucose overload in yolk has little effect on the long-term modulation of carbohydrate metabolic genes in zebrafish (*Danio rerio*)." Journal of Experimental Biology **217**(7): 1139-1149.

Rønnestad, I., W. M. Koven, A. Tandler, M. Harel and H. J. Fyhn (1994). "Energy metabolism during development of eggs and larvae of gilthead sea bream (Sparus aurata)." Marine Biology **120**(2): 187-196.

Rosenlund, G., G. Corraze, M. Izquierdo and B. E. Torstensen (2010). The effects of fish oil replacement on nutritional and organoleptic qualities of farmed fish. Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. G. M. Turchini, W. K. Ng and D. R. Tocher. Boca Raton, Florida, USA, CRC Press**:** 487-522.

Rowley, A. F., J. Knight, P. Lloyd-Evans, J. W. Holland and P. J. Vickers (1995). "Eicosanoids and their role in immune modulation in fish—a brief overview." Fish & Shellfish Immunology **5**(8): 549-567.

SACN, U. (2004). Advice on fish consumption: benefits & risks, Istituto Superiore di Sanit.

Saera-Vila, A., J. A. Calduch-Giner, P. Gómez-Requeni, F. Médale, S. Kaushik and J. Pérez-Sánchez (2005). "Molecular characterization of gilthead sea bream (*Sparus aurata*) lipoprotein lipase. Transcriptional regulation by season and nutritional condition in skeletal muscle and fat storage tissues." Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology **142**(2): 224-232.

Saleh, R., M. Betancor, J. Roo, V. Benítez-Dorta, M. Zamorano, J. Bell and M. Izquierdo (2014). "Effect of krill phospholipids versus soybean lecithin in microdiets for gilthead seabream (Sparus aurata) larvae on molecular markers of antioxidative metabolism and bone development." Aquaculture Nutrition.

Santos, C. R. A., D. M. Power, P. Kille, L. Llewellyn, V. Ramsurn, T. Wigham and G. E. Sweeney (1997). "Cloning and Sequencing of a Full-Length Sea Bream (*Sparus aurata*) β-Actin cDNA." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **117**(2): 185-189.

Sargent, J., G. Bell, L. McEvoy, D. Tocher and A. Estevez (1999). "Recent developments in the essential fatty acid nutrition of fish." Aquaculture **177**(1-4): 191-199.

Sargent, J., D. Tocher and J. Bell (2002). The lipids. Fish Nutrition. J. E. Halver and R. W. Hardy. San Diego, USA, Academic Press**:** 181-257.

Sargent, J. R., J. G. Bell, M. V. Bell, R. J. Henderson and D. R. Tocher (1995). "Requirement criteria for essential fatty acids." Journal of Applied Ichthyology **11**(3-4): 183-198.

Sarkar, D. (2008). Lattice: multivariate data visualization with R. New York, USA, Springer Science & Business Media.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative CT method." Nature Protocols **3**(6): 1101-1108.

Secombes, C. J., L. J. Hardie and G. Daniels (1996). "Cytokines in fish: an update." Fish & Shellfish Immunology **6**(4): 291-304.

Seiliez, I., S. Panserat, G. Corraze, S. Kaushik and P. Bergot (2003). "Cloning and nutritional regulation of a Δ6-desaturase-like enzyme in the marine teleost gilthead seabream (*Sparus aurata*)." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **135**(3): 449-460.

Sepulcre, M. P., G. López-Castejón, J. Meseguer and V. Mulero (2007). "The activation of gilthead seabream professional phagocytes by different PAMPs underlines the behavioural diversity of the main innate immune cells of bony fish." Molecular Immunology **44**(8): 2009-2016.

Serhan, C. N. (2006). "Novel Chemical Mediators in the Resolution of Inflammation: Resolvins and Protectins." Anesthesiology Clinics of North America **24**(2): 341-364.

Shepherd, J. and E. Bachis (2014). "Changing supply and demand for fish oil." Aquaculture Economics & Management **18**(4): 395-416.

Siemelink, M., A. Verhoef, J. A. Dormans, P. N. Span and A. H. Piersma (2002). "Dietary fatty acid composition during pregnancy and lactation in the rat programs growth and glucose metabolism in the offspring." Diabetologia **45**(10): 1397-1403.

Simopoulos, A. (2000). "Human requirement for N-3 polyunsaturated fatty acids." Poultry science **79**(7): 961-970.

Simopoulos, A. P. (2008). "The Importance of the Omega-6/Omega-3 Fatty Acid Ratio in Cardiovascular Disease and Other Chronic Diseases." Experimental Biology and Medicine **233**(6): 674-688.

Sinclair, K. D., C. Allegrucci, R. Singh, D. S. Gardner, S. Sebastian, J. Bispham, A. Thurston, J. F. Huntley, W. D. Rees and C. A. Maloney (2007). "DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status." Proceedings of the National Academy of Sciences **104**(49): 19351-19356.

Skiba-Cassy, S., S. Panserat, M. Larquier, K. Dias, A. Surget, E. Plagnes-Juan, S. Kaushik and I. Seiliez (2013). "Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (Oncorhynchus mykiss)." British Journal of Nutrition **109**(8): 1359-1372.

Sokal, R. R. and F. J. Rohlf (1969). The principles and practice of statistics in biological research, WH Freeman and company San Francisco:.

Song, C., X. Li, B. E. Leonard and D. F. Horrobin (2003). "Effects of dietary n-3 or n-6 fatty acids on interleukin-1β-induced anxiety, stress, and inflammatory responses in rats." Journal of Lipid Research **44**(10): 1984-1991.

Stoffel, W., B. Holz, B. Jenke, E. Binczek, R. Günter, C. Kiss, L. Karakesisoglou, M. Thevis, A. Weber, S. Arnhold and K. Addicks (2008). "Delta6-desaturase (FADS2) deficiency unveils the role of omega3- and omega6-polyunsaturated fatty acids." The EMBO journal **27**(17): 2281-2292.

Su, Z., L. Han and Z. Zhao (2011). "Conservation and divergence of DNA methylation in eukaryotes: new insights from single base-resolution DNA methylomes." Epigenetics **6**.

Symonds, M. E. and H. Budge (2009). "Nutritional models of the developmental programming of adult health and disease." Proceedings of the Nutrition Society **68**(2): 173-178.

Tacon, A. G. J. and M. Metian (2008). "Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects." Aquaculture **285**(1-4): 146-158.

Thanuthong, T., D. S. Francis, S. P. S. D. Senadheera, P. L. Jones and G. M. Turchini (2011). "LC-PUFA Biosynthesis in Rainbow Trout is Substrate Limited: Use of the Whole Body Fatty Acid Balance Method and Different 18:3n-3/18:2n-6 Ratios." Lipids **46**(12): 1111-1127.

Thomassen, M. S., D. Rein, G. M. Berge, T.-K. Østbye and B. Ruyter (2012). "High dietary EPA does not inhibit Δ5 and Δ6 desaturases in Atlantic salmon (Salmo salar L.) fed rapeseed oil diets." Aquaculture **360-361**: 78-85.

Tocher, D. R. (2003). "Metabolism and functions of lipids and fatty acids in teleost fish." Reviews in Fisheries Science **11**(2): 107-184.

Tocher, D. R. (2010). "Fatty acid requirements in ontogeny of marine and freshwater fish." Aquaculture Research **41**(5): 717-732.

Tocher, D. R. (2015). "Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective." Aquaculture.

Tocher, D. R., A. J. Fraser, J. R. Sargent and J. C. Gamble (1985). "Fatty acid composition of phospholipids and neutral lipids during embryonic and early larval development in Atlantic herring (Clupea harengus, L.)." Lipids **20**(2): 69-74.

Tocher, D. R., A. J. Fraser, J. R. Sargent and J. C. Gamble (1985). "Lipid class composition during embryonic and early larval development in Atlantic herring (Clupea harengus, L.)." Lipids **20**(2): 84-89.

Tocher, D. R. and C. Ghioni (1999). "Fatty acid metabolism in marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream (*Sparus aurata*) cells." Lipids **34**(5): 433-440.

Tocher, D. R., X. Zheng, C. Schlechtriem, N. Hastings, J. R. Dick and A. J. Teale (2006). "Highly unsaturated fatty acid synthesis in marine fish: cloning, functional characterization, and

nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.)." Lipids **41**(11): 1003-1016.

Todd, S. E., M. H. Oliver, A. L. Jaquiery, F. H. Bloomfield and J. E. Harding (2009). "Periconceptional undernutrition of ewes impairs glucose tolerance in their adult offspring." Pediatric Research **65**(4): 409-413.

Torrecillas, S., D. Mompel, M. J. Caballero, D. Montero, D. Merrifield, A. Rodiles, L. Robaina, M. J. Zamorano, V. Karalazos, S. Kaushik and M. Izquierdo (2017). "Effect of fishmeal and fish oil replacement by vegetable meals and oils on gut health of European sea bass (Dicentrarchus labrax)." Aquaculture **468**: 386-398.

Torrecillas, S., L. Robaina, M. J. Caballero, D. Montero, G. Calandra, D. Mompel, V. Karalazos, S. Kaushik and M. S. Izquierdo (2017). "Combined replacement of fishmeal and fish oil in European sea bass (*Dicentrarchus labrax*): Production performance, tissue composition and liver morphology." Aquaculture **474**: 101-112.

Torrecillas, S., L. Román, F. Rivero-Ramírez, M. J. Caballero, C. Pascual, L. Robaina, M. S. Izquierdo, F. Acosta and D. Montero (2017). "Supplementation of arachidonic acid rich oil in European sea bass juveniles (Dicentrarchus labrax) diets: Effects on leucocytes and plasma fatty acid profiles, selected immune parameters and circulating prostaglandins levels." Fish & Shellfish Immunology **64**: 437-445.

Torstensen, B., M. Espe, M. Sanden, I. Stubhaug, R. Waagbø, G.-I. Hemre, R. Fontanillas, U. Nordgarden, E. Hevrøy and P. Olsvik (2008). "Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends." Aquaculture **285**(1): 193-200.

Torstensen, B., W. Ng and D. Tocher (2010). The Effects of Fish Oil Replacement on Lipid Metabolism of Fish. Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. G. M. Turchini, W. K. Ng and D. R. Tocher. Boca Raton, Florida, USA, CRC Press.

Torstensen, B. E., J. G. Bell, G. Rosenlund, R. J. Henderson, I. E. Graff, D. R. Tocher, O. Lie and J. R. Sargent (2005). "Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid composition and sensory quality by replacing fish oil with a vegetable oil blend." Journal of Agricultural and Food Chemistry **53**(26): 10166-10178.

Turchini, G. M., T. Mentasti, L. Froyland, E. Orban, F. Caprino, V. M. Moretti and F. Valfre (2003). "Effects of alternative dietary lipid sources on performance, tissue chemical composition, mitochondrial fatty acid oxidation capabilities and sensory characteristics in brown trout (*Salmo trutta* L.)." Aquaculture **225**(1-4): 251-267.

Turchini, G. M., B. E. Torstensen and W. K. Ng (2009). "Fish oil replacement in finfish nutrition." Reviews in Aquaculture **1**(1): 10-57.

Turkmen, S., P. L. Castro, M. J. Caballero, C. M. Hernández-Cruz, R. Saleh, M. J. Zamorano, J. Regidor and M. Izquierdo (2017). "Nutritional stimuli of gilthead seabream (*Sparus aurata*) larvae by dietary fatty acids: effects on larval performance, gene expression and neurogenesis." Aquaculture Research **48**(1): 202-213.

Turkmen, S. and M. Izquierdo (2014). *Sparus aurata* as a model for nutritional reprogramming of marine fish: Effectiveness of different developmental windows. EPICONCEPT Workshop

2014 Epigenomic Toolbox: from Methods to Models, Las Palmas, Spain, University of Las Palmas and the COST Action FA1201.

Turkmen, S., M. J. Zamorano, H. Fernández-Palacios, C. M. Hernández-Cruz, D. Montero, L. Robaina and M. Izquierdo (2017). "Parental nutritional programming and a reminder during juvenile stage affect growth, lipid metabolism and utilisation in later developmental stages of a marine teleost, the gilthead sea bream (*Sparus aurata*)." British Journal of Nutrition **118**(7): 500-512.

Uauy, R., C. Corvalan and A. D. Dangour (2009). "Rank Prize Lecture Global nutrition challenges for optimal health and well-being." Proceedings of the Nutrition Society **68**(01): 34-42.

Vagner, M., J. H. Robin, J. L. Zambonino Infante and J. Person-Le Ruyet (2007). "Combined effects of dietary HUFA level and temperature on sea bass (*Dicentrarchus labrax*) larvae development." Aquaculture **266**(1-4): 179-190.

Vagner, M., J. H. Robin, J. L. Zambonino-Infante, D. R. Tocher and J. Person-Le Ruyet (2009). "Ontogenic effects of early feeding of sea bass (*Dicentrarchus labrax*) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways." British Journal of Nutrition **101**(10): 1452-1462.

Vagner, M. and E. Santigosa (2011). "Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: A review." Aquaculture **315**(1-2): 131-143.

Vagner, M., J. L. Zambonino Infante, J. H. Robin and J. Person-Le Ruyet (2007). "Is it possible to influence European sea bass (*Dicentrarchus labrax*) juvenile metabolism by a nutritional conditioning during larval stage?" Aquaculture **267**(1-4): 165-174.

Vandamme, T. F. (2014). "Use of rodents as models of human diseases." Journal of pharmacy & bioallied sciences **6**(1): 2-9.

Vassallo-Agius, R., T. Watanabe, G. Yoshizaki, S. Satoh and Y. Takeuchi (2001). "Quality of eggs and spermatozoa of rainbow trout fed an n-3 essential fatty aciddeficient diet and its effects on the lipid and fatty acid components of eggs, semen and livers." Fisheries Science **67**(5): 818-827.

Vera, L. M., C. Metochis, J. F. Taylor, M. Clarkson, K. H. Skjærven, H. Migaud and D. R. Tocher (2017). "Early nutritional programming affects liver transcriptome in diploid and triploid Atlantic salmon, *Salmo salar*." BMC Genomics **18**: 886.

Vessby, B., I. B. Gustafsson, S. Tengblad, M. Boberg and A. Andersson (2002). "Desaturation and elongation of fatty acids and insulin action." Annals of the New York Academy of Sciences **967**(1): 183-195.

Vestergren, A. S., S. Trattner, J. Pan, P. Johnsson, A. Kamal-Eldin, E. Brännäs, A. A. Moazzami and J. Pickova (2012). "The effect of combining linseed oil and sesamin on the fatty acid composition in white muscle and on expression of lipid-related genes in white muscle and liver of rainbow trout (*Oncorhynchus mykiss*)." Aquaculture International **21**(4): 843-859.

Wang, C., W. S. Harris, M. Chung, A. H. Lichtenstein, E. M. Balk, B. Kupelnick, H. S. Jordan and J. Lau (2006). "n–3 Fatty acids from fish or fish-oil supplements, but not α-linolenic acid,

benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review." The American Journal of Clinical Nutrition **84**(1): 5-17.

Waterland, R. A. and R. L. Jirtle (2003). "Transposable elements: Targets for early nutritional effects on epigenetic gene regulation." Molecular and Cellular Biology **23**(15): 5293-5300.

Whelan, J. and C. Rust (2006). "Innovative dietary sources of n-3 fatty acids." Annu Rev Nutr **26**: 75-103.

Wolff, G. L., R. L. Kodell, S. R. Moore and C. A. Cooney (1998). "Maternal epigenetics and methyl supplements affect agouti gene expression in A(vy)/a mice." Faseb Journal **12**(11): 949-957.

World Health Organisation. (2018). "The top 10 causes of death." Retrieved 12.12, 2018, from https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death.

Xie, F., P. Xiao, D. Chen, L. Xu and B. Zhang (2012). "miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs." Plant Mol Biol.

Xu, H., X. Dong, Q. Ai, K. Mai, W. Xu, Y. Zhang and R. Zuo (2014). "Regulation of Tissue LC-PUFA Contents, Δ6 Fatty Acyl Desaturase (FADS2) Gene Expression and the Methylation of the Putative FADS2 Gene Promoter by Different Dietary Fatty Acid Profiles in Japanese Seabass (*Lateolabrax japonicus*)." PloS one **9**(1): e87726.

Xue, X., S. M. Hixson, T. S. Hori, M. Booman, C. C. Parrish, D. M. Anderson and M. L. Rise (2015). "Atlantic salmon (*Salmo salar*) liver transcriptome response to diets containing Camelina sativa products." Comparative Biochemistry and Physiology, Part D: Genomics and Proteomics **14**: 1-15.

Yaqoob, P. (2004). "Fatty acids and the immune system: from basic science to clinical applications." Proc Nutr Soc **63**(1): 89-104.

Yaqoob, P. and P. C. Calder (2007). "Fatty acids and immune function: new insights into mechanisms." British Journal of Nutrition **98**(S1): S41-S45.

Yılmaz, H. A., G. Corraze, S. Panserat and O. T. Eroldoğan (2016). "Effects of alternate feeding with different lipid sources on fatty acid composition and bioconversion in European sea bass (*Dicentrarchus labrax*)." Aquaculture **464**: 28-36.

Zammit, V. A. (1999). "The malonyl-CoA–long-chain acyl-CoA axis in the maintenance of mammalian cell function." Biochemical Journal **343**(3): 505-515.

Zenk, F., E. Loeser, R. Schiavo, F. Kilpert, O. Bogdanović and N. Iovino (2017). "Germ line–inherited H3K27me3 restricts enhancer function during maternal-to-zygotic transition." Science **357**(6347): 212-216.

Zheng, X., I. Seiliez, N. Hastings, D. R. Tocher, S. Panserat, C. A. Dickson, P. Bergot and A. J. Teale (2004). "Characterization and comparison of fatty acyl Delta6 desaturase cDNAs from freshwater and marine teleost fish species." Comp Biochem Physiol B Biochem Mol Biol **139**(2): 269-279.

ANNEX

Publications and awards/scholarships received during the PhD project

SCI articles

1. **Turkmen S.**, Hernández-Cruz C.M., Zamorano M.J., Fernández-Palacios H., Montero D., Afonso J.M., Izquierdo M. (Minor revisions) LC-PUFA profiles in parental diets induced long-term effects on growth, fatty acid profiles and expression of selected PUFA metabolism and immune system related genes in gilthead seabream offspring. **British Journal of Nutrition**, In press.
2. **Turkmen S.**, Zamorano M.J., Fernández-Palacios H., Hernández-Cruz C.M., Montero D., Robaina L., Izquierdo M. (2017) Parental nutritional programming and a reminder during juvenile stage affect growth, lipid metabolism and utilisation in later developmental stages of a marine teleost, the gilthead sea bream (*Sparus aurata*). **British Journal of Nutrition**, 118, 500-512.
3. Izquierdo M., **Turkmen S.**, Montero D., Zamorano M.J., Afonso J.M., Karalazos V., Fernández-Palacios H. (2015) Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. **Aquaculture,** 449, 18-26.
4. Chapter 6. To be submitted…
5. Chapter 7. To be submitted…

Oral presentation at international congresses

1. **Turkmen S.**, Xu H., Fernández-Palacios H., Zamorano M.J., Izquierdo M. (2018) Nutritional programming of gilthead sea bream: Improvements towards better utilization of low n-3 HUFA diets. Oral presentation, **International Symposium on Fish Nutrition and Feeding 2018**, Las Palmas, Spain.
2. **Turkmen S.**, Xu H., Saleh R., Hernández-Cruz C.M., Zamorano M.J., Izquierdo M. (2017) Nutritional programming through broodstock nutrition: effect of low fish meal and/or fish oil diets on offspring larval performance: growth, biochemical composition and gene expression. Oral presentation, **Aquaculture Europe 2017**, Dubrovnik, Croatia.

3. **Turkmen S.** (2017) Can we prepare the fish for their future environment? - Nutritional programming. Oral presentation, **Aquaculture Europe 2017**, Dubrovnik, Croatia.
4. **Turkmen S.**, Hernández-Cruz C.M., Zamorano M.J., Sarih S., Xu H., Izquierdo M. (2017) Nutritional programming through broodstock diets in gilthead sea bream; effects on spawning quality, larval growth and long-term effects on the offspring Oral presentation, **Larvi 2017**, Ghent, Belgium.
5. **Turkmen S.**, Fernández-Palacios H., Hernández-Cruz C.M., Zamorano M.J., Izquierdo M. (2016) Effects of selection of broodstock by a genetic biomarker (delta-6-desaturase) and nutritional programming by vegetable oil inclusion in the broodstock diets: effects on reproduction quality and larval growth of gilthead sea bream *Sparus aurata*. Poster presentation, **Aquaculture Europe 2016**, Edinburgh, Scotland.
6. **Turkmen S.**, Fernández-Palacios H., Hernández-Cruz C.M., Zamorano M.J., Izquierdo M. (2016) Testing long-term effects of parental nutritional programming with vegetable oils and a "reminder" diet during juvenile stage on growth and lipid metabolism related genes in gilthead sea bream (*Sparus aurata*). Oral presentation, **International Symposium on Fish Nutrition and Feeding 2016**, Sun Valley, Idaho, USA.
7. **Turkmen S.**, Izquierdo M. (2015) Testing long-term effects of parental nutritional programming and a "reminder" juvenile programing on lipid metabolism and growth in *Sparus aurata*. Poster presentation, **Epigenetics and Preconception Environment**, Dubrovnik, Croatia.
8. **Turkmen S.**, Izquierdo M. (2014) *Sparus aurata* as a model for nutritional reprogramming of marine fish: Effectiveness of different developmental windows. Oral presentation, **Epigenetics and Preconception Environment**, Las Palmas, Spain.

Oral presentations in other meetings

1. **Turkmen S.**, (2017) Programación nutricional y epigenetica en peces. Oral presentation, **Dia Mundial de Acuicultura**, Las Palmas, Spain.

2. **Turkmen S.**, (2017) Nutritional programing in Fish: "*Sparus aurata* as a model for nutritional programing of marine fish". Oral presentation, ECOAQUA Institute **Epigenetic Meeting**, Las Palmas, Spain.

Collaborative works presented by others at international meetings

1. Xu H., **Turkmen S**., Ferosekhan S., Fernández-Palacios H., Afonso J.M., Izquierdo M. (2018) Effect of low fish meal and fish oil diet on growth performance, hepatic fatty acid composition and *fads2* expression of juvenile gilthead sea bream (*Sparus aurata*) from nutritional programmed broodstock Oral presentation, International Symposium on Fish Nutrition and Feeding, Las Palmas, Spain.
2. Ferosekhan S., Xu H., **Turkmen S**., Fernández-Palacios H., Afonso J.M., Gomez A., Kaushik S., Izquierdo M. (2018) Influence of dietary fatty acid profile on reproductive performance in gilthead seabream, Sparus aurata broodstock selected for high or low fads2 expression Poster presentation, International Symposium on Fish Nutrition and Feeding, Las Palmas, Spain.
3. Saleh R., Burri L., Benitez-Santana T., **Turkmen S**., Castro P., Izquierdo M. (2018) Dietary Krill Meal Inclusion Contributes to Better Growth Performance of Gilthead Seabream Juveniles Poster presentation, Dia Mundial de Acuicultura, Las Palmas, Spain.
4. Xu H., **Turkmen S.,** Sarih S., Fernández-Palacios H., Zamorano M.J., Afonso J.M., Izquierdo M. (2017) Nutritional programming through broodstock nutrition: effect of low fishmeal and/or fish oil diets on spawning quality of broodstock with high fads2 expression. Poster presentation, Aquaculture Europe, Dubrovnik, Croatia.
5. Saleh R., Betancor M., **Turkmen S.,** Izquierdo M. (2017) Dietary phospholipid type and level in microdiets for gilthead sea-bream larvae: effects on histological changes in intestine and liver. Poster presentation, larvi 2017, Ghent, Belgium.
6. Eroldogan O. T., Ocal N. N., Yilmaz H. A., **Turkmen S.,** Olculu A. (2016) The effects of short starvation and refeeding on lipid metabolism in European sea bass Dicentrarchus labrax at different temperatures. Poster presentation, Aquaculture Europe, Edinburgh, Scotland.

Awards and scholarships

1. 2 times, Epiconcept COST action, grant to attend congresses and workshops financed by European Union.
2. Grant to make oral presentation Larvi17 symposium, organized by Ghent University.
3. Grant to make oral presentation at Aquaculture Europe 2017 granted by European Aquaculture Society's student group.
4. Best students' oral presentation at International Symposium on Fish Nutrition and Feeding, Las Palmas, Spain.
5. Fully-financed by CIHEAM to make an oral presentation at student and young Researchers forum at Bari, Italy.